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The Journal of Clinical Investigation

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The Journal of Clinical Investigation

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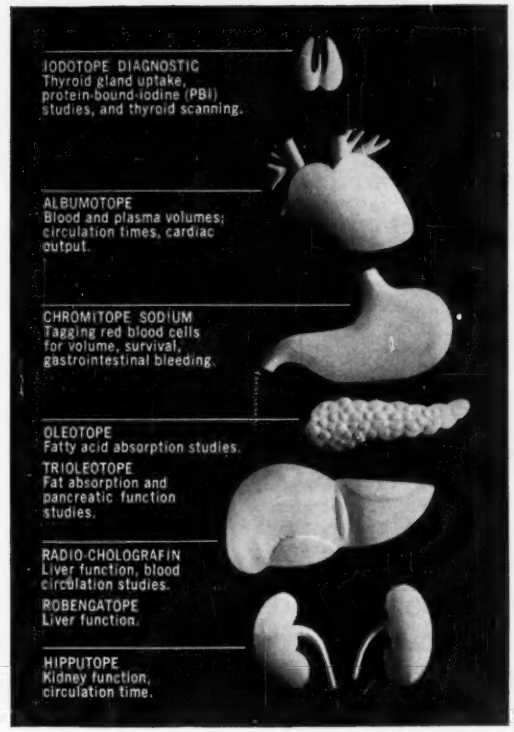

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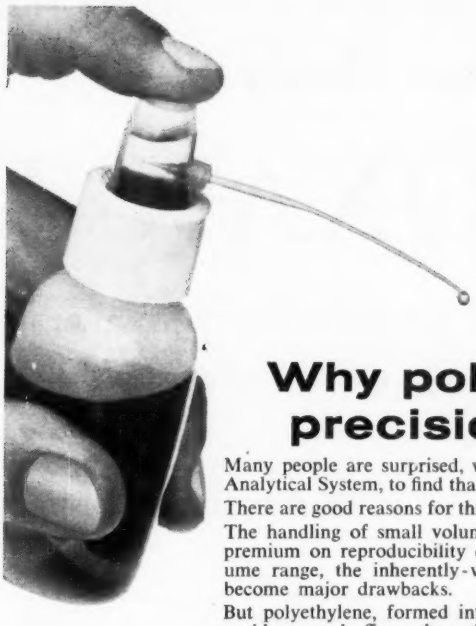

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CYTOTOXIC AGENT for palliative chemotherapy of certain types of malignant neoplasms

Cytoxan demonstrated therapeutic advantage over certain other agents in a recent 12-month study* of 130 patients, most of whom were refractory to previous treatment:

DISEASE	NUMBER OF PATIENTS	RESULTS				INADEQUATE TRIAL
		GOOD	FAIR	TRANSIENT	FAILURE	
Lymphoma	**74**	**34**	**3**	**5**	**23**	**9**
Hodgkin's Disease	29	10	3	4	9	3
Lymphosarcoma	21	15	0	0	3	3
Multiple Myeloma	16	9	0	0	4	3
Reticulum Cell Disease	8	0	0	1	7	0
Leukemia	**23**	**10**	**0**	**0**	**8**	**5**
Chronic Lymphatic Leukemia	8	4	0	0	3	1
Acute Monoblastic Leukemia	11	5	0	0	3	3
Acute Myeloblastic Leukemia	4	1	0	0	2	1
Carcinoma (Breast, Lung, and Solid Tumors)	**29**	**2**	**1**	**1**	**23**	**2**
Miscellaneous (Mycosis Fungoides, Psoriasis)	**4**	**0**	**0**	**1**	**3**	**0**
Total	130	46	4	7	57	16

Adapted from Wall, R. L., and Conrad, F. G.*

Note that the neoplastic disorders most responsive to Cytoxan were lymphosarcoma, multiple myeloma, Hodgkin's disease, and chronic lymphatic leukemia. Occasionally, good results were observed in acute monocytic leukemia and carcinoma of the breast.

Other advantages noted in this study*

• multiple routes of administration, permitting prolonged maintenance therapy • lack of latency period for bone marrow depression • failure to produce significant thrombocytopenia • potential therapeutic effect in diseases usually unresponsive to other mustard compounds (*e.g.*, myeloma).

*Wall, R. L., and Conrad, F. G.: Arch. Int. Med. *108*:456-482, 1961.

INDICATIONS: Cytoxan is valuable for palliative therapy of certain malignant neoplasms, particularly some of those arising in the reticuloendothelial and hematopoietic systems and certain solid tumors.

Types of cancer which have proved relatively more susceptible or more resistant to Cytoxan therapy may be grouped as follows:

Group I: Neoplasms relatively susceptible to Cytoxan
- Hodgkin's disease
- Lymphomas: lymphosarcoma; giant follicular lymphoma; reticulum cell sarcoma
- Leukemia: acute; chronic
- Mycosis fungoides

Group II: Neoplasms relatively resistant to Cytoxan
- Malignant neoplasms of the breast and the ovary*
- Malignant neoplasms of the lung, the gastrointestinal tract and the genitourinary system, including the cervix and the uterus
- Malignant neoplasms of miscellaneous origin
- Malignant melanomas

*Malignant tumors of these organs are somewhat more susceptible to Cytoxan therapy than are the others included in this group.

DOSAGE: *For neoplasms relatively susceptible to Cytoxan* – Patients with lymphomas and other neoplasms believed to be relatively susceptible to Cytoxan therapy are given an initial dose of 2 to 3 mg./Kg./day intravenously. White blood counts and platelet determinations should be made daily or twice weekly and the dosage adjusted accordingly. Intravenous infusions should be continued for at least 6 days unless otherwise indicated. A leukopenia of between 1500 and 5000 cells per cu. mm. (or lower) may be expected between the tenth and fourteenth day. In the presence of a leukopenia of less than 2000/cu. mm. Cytoxan should be discontinued until the white cell count returns to 2000 to 5000 (usually within a week). Dosage is subsequently adjusted as indicated by the patient's objective response and the leukocyte count. If the patient is subjectively improved, if the size of the tumor has decreased, or if the white cells are satisfactorily maintained between 2000 and 5000/cu. mm. oral dosage may be instituted equivalent to intravenous dosage.

Thrombocytopenia is rarely observed on this regimen. If platelet counts of less than 100,000/cu. mm. are observed, the patient should be watched carefully. If platelets continue to decrease, Cytoxan should be discontinued.

The patient who has had previous treatment with alkylating agents, or x-ray, or is debilitated may be more susceptible to bone marrow depression, and initial Cytoxan doses should be more conservative than the above. Such patients should have more frequent hematologic evaluation. Good medical practice demands access to a reliable hematologic laboratory when using Cytoxan.

For neoplasms relatively resistant to Cytoxan – Patients with carcinomas and other malignant neoplasms believed to be less susceptible to Cytoxan therapy are given a dose of 4 to 8 mg./Kg./day intravenously. Unless there are indications to the contrary, this dose is continued for 6 days, then stopped. Leukopenia usually ensues on the tenth to fourteenth day after the first dose of Cytoxan. Thrombocyte reduction is not common, and platelets may actually increase. The leukocyte count promptly returns toward normal levels in most cases, and as it begins to increase, sufficient Cytoxan is administered to maintain it near 2000 to 5000/cu. mm. This may be accomplished by two intravenous injections weekly, or by oral administration, or by a combination of both routes. An oral dosage of 50 to 200 mg. daily or an intravenous injection of 5 mg./Kg. twice weekly will usually suffice.

The platelet and leukocyte counts should be followed carefully, and the prior treatment history of patients carefully evaluated as delineated above.

Leukopenia as a guide to adequacy of dosage – The best objective measure for dosage seems to be the number of circulating white blood cells. This is used as an index of the activity of the hematopoietic system, especially the bone marrow. The mechanism by which Cytoxan causes a reduction in the level of white blood cells is not known, but cessation of dosage results in an increase in the level, indicating that the hematopoietic system had not been permanently affected. When large doses (8 mg./Kg./day for 6 days) are given initially, the white cell count falls rapidly. Following the cessation of the 6-day course, the white cells may continue to decline for as long as 8 days and then increase. The reduction of the white cell count during Cytoxan therapy and its subsequent increase when therapy is discontinued can be repeated in the same patient. Maximal reduction in leukocyte count indicates the maximal permissible Cytoxan level for therapeutic effect. Leukopenic patients must be watched carefully for evidence of infection.

Total white blood cell and thrombocyte counts should be obtained 2 or more times weekly in order to evaluate therapy and to adjust dosage.

SIDE EFFECTS: Although Cytoxan is related to nitrogen mustard, it has no vesicant effect on tissue. It does not traumatize the vein when injected intravenously, nor does it cause any localized tissue reaction following extravasation. It may be administered intravenously, intramuscularly, intraperitoneally, intrapleurally or directly into the tumor, when indicated. It is apparently active by each of these routes.

Nausea and vomiting are common and depend on dose and on individual susceptibility. However, many investigators accept the nausea and vomiting in favor of maintaining maximal therapy. The vomiting can be controlled with antiemetic agents.

Alopecia is a frequent side reaction to Cytoxan therapy. It has been observed in 28% of the patients studied in this country. The incidence is greater with larger doses. The loss of hair may first be noted about the 21st day of therapy and may proceed to alopecia totalis. This effect is reversed following discontinuance of Cytoxan; during reduced maintenance therapy, hair may reappear. It is essential to advise the patient in advance concerning this effect of the drug.

Dizziness of short duration and of minor degree has occasionally been reported.

Leukopenia is an expected effect and can be used as a guide to therapy. Thrombocytopenia may occur, especially after large doses. The leukocyte or platelet counts of an occasional patient may fall precipitously after even small doses of Cytoxan, as with all alkylating agents. The drug should be discontinued in such patients and reinstituted later at lower dosage after satisfactory hematologic recovery has occurred. Prior treatment with x-ray or with other chemotherapeutic agents frequently causes an earlier or exaggerated leukopenia or thrombocytopenia after Cytoxan medication. Only rarely has there been a report of erythrocyte or hemoglobin reduction.

ADMINISTRATION: Add 5 cc. sterile water (Water for Injection, U.S.P.) to 100 mg. of Cytoxan in the sterile vial (add 10 cc. to 200 mg. vial). Shake, allow to stand until clear, remove with sterile syringe and needle and inject.

The freshly prepared solution of Cytoxan may be administered intravenously, intramuscularly, intraperitoneally, intrapleurally, or directly into the tumor. The solution should be administered promptly after being made but is satisfactory for use for three hours after preparation.

If the patient is receiving a parenteral infusion, the Cytoxan solution may be injected into the rubber tubing if the solution is glucose or saline.

No thrombosis or thrombophlebitis has been reported from injections of Cytoxan. Extravasation of the drug into the subcutaneous tissues does not result in local reactions.

PRECAUTIONS: Cytoxan should not be given to any person with a severe leukopenia, thrombocytopenia, or bone marrow infiltrated with malignant cells. It may be given with suitable precautions to patients who have had recent x-ray treatment, recent treatment with a cytotoxic agent, a surgical procedure within 2 to 3 weeks, or debilitated patients.

AVAILABILITY: Cytoxan is available as follows:

Cytoxan for Injection, 100 mg., a sterile dry-filled vial containing 100 mg. cyclophosphamide and 45 mg. sodium chloride. Packaged, 12 vials per carton.

Cytoxan for Injection, 200 mg., a sterile dry-filled vial containing 200 mg. cyclophosphamide and 90 mg. sodium chloride. Packaged, 12 vials per carton.

Cytoxan Tablets for oral administration, 50 mg., white, round tablets, flecked with blue for easy identification. Packaged, 100 tablets per bottle.

For a copy of the Cytoxan brochure, or other additional information on Cytoxan, communicate directly with the Medical Department, Mead Johnson Laboratories, Evansville 21, Indiana.

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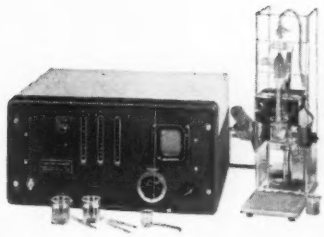

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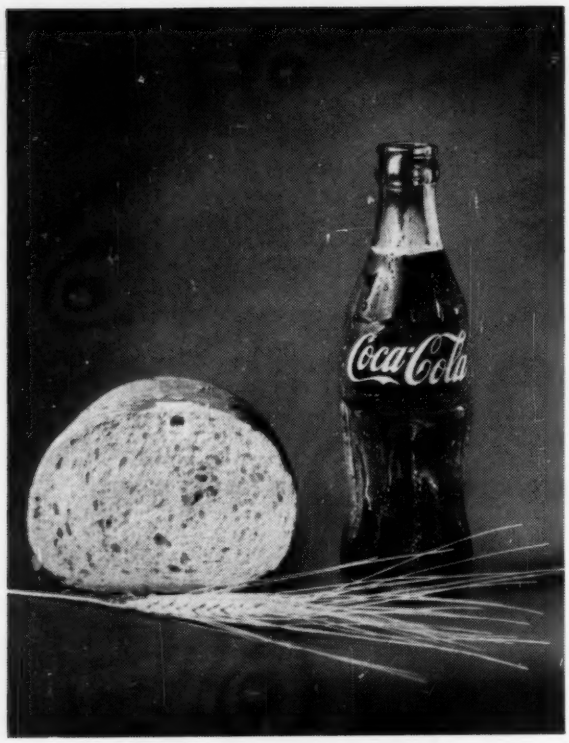

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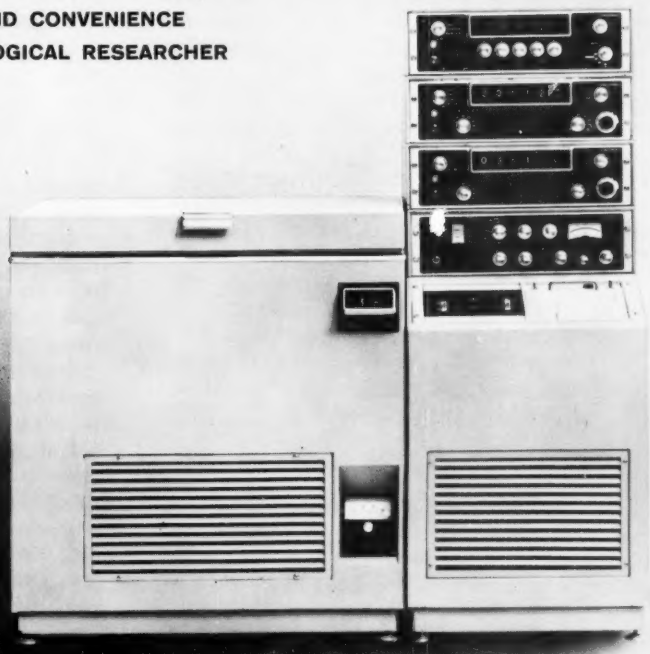

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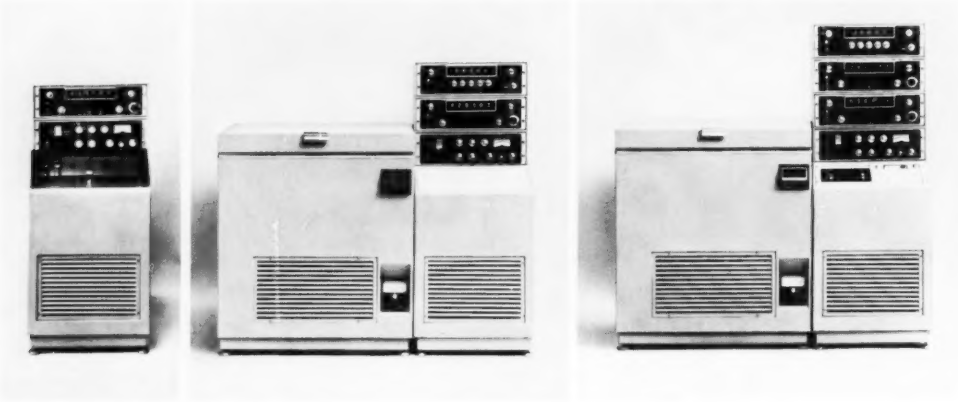

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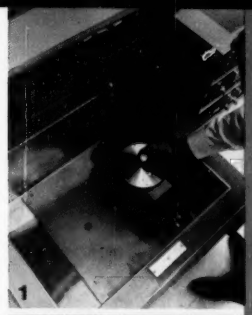
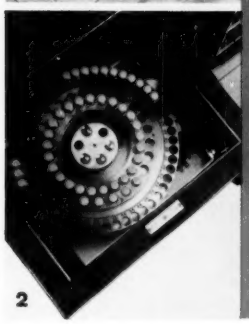
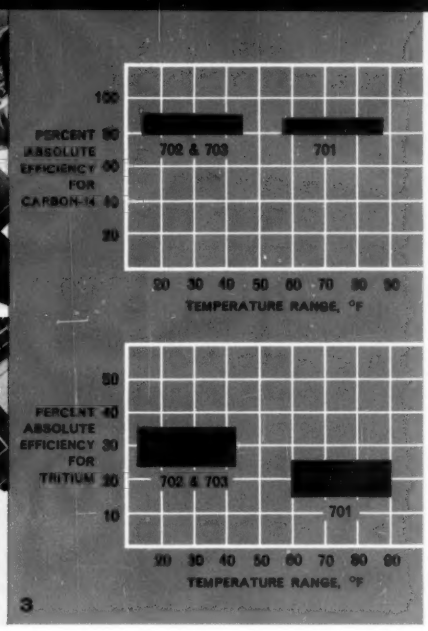
The Series 700—compatible with your present standard scaling instruments—easily accommodates to growth of research programs and equipment needs. The interchangeability of the unique dual detector, elevator assembly, solid-state electronics, and cabinetry permits the small laboratory to advance from one system to another as the budget grows—to go from *manual* operation at room temperatures with the 701, to manual *temperature-controlled* operation with the 702, and on to *automatic* temperature-controlled operation with the 703. Larger laboratories, requiring utmost precision for large numbers of samples, can obtain the 703 system with sophisticated, automatic readout — provided by Nuclear-Chicago's new Logic Series scalers, timer, and printer.

In all three systems, independent two-channel selection permits overlapping or separated channel counting. True double pulse resolution of less than one microsecond allows high speed counting with extremely small resolution losses. New, high gain photomultiplier tubes permit use of low electronic gain, providing vastly improved overload recovery characteristics while eliminating system "paralysis." Now, isotopes of widely differing energies and samples with countrates varying from 500 cpm to 1,000,000 cpm can be counted alternately without gain shifts or memory effects causing loss of reproducibility.

MODEL 701 Liquid Scintillation System is chosen for economical, room temperature counting of carbon-14 and tritium. It is a modestly priced, high efficiency basic system with components common to all 700 Series models. The compact console houses a motor-driven elevator to position the sample in an optical cell; a dual-detector assembly; stabilized high voltage supply; fast coincidence circuitry; and a high speed, dual channel pulse-height analyzer. The detector assembly consists of high efficiency dual photomultiplier tubes shielded from external radiation and ambient temperature changes by steel and lead. A precision, automatically operated shutter eliminates light exposure to the photomultiplier tubes and permits use of the 701







1) Sample being placed on elevator of detector assembly in 702 controlled temperature chamber. Ample space is provided in cooling chamber for storage of samples.

2) Automatic sample changer in the 703 cooling chamber. Sample bottles are located at the edge of the circular table. Removable racks for additional samples and scintillation standards are provided.

3) Charts of 700 Series efficiency ranges obtained in integral counting. Sample used was labeled toluene with PPO and POPOP. For carbon-14 the 702 and 703 yield efficiencies of 80-90%, while the 701 provides an efficiency range of 80-85%. For tritium the 702 and 703 provide an efficiency range of 25-35%, while the 701 efficiency is 15-25%.

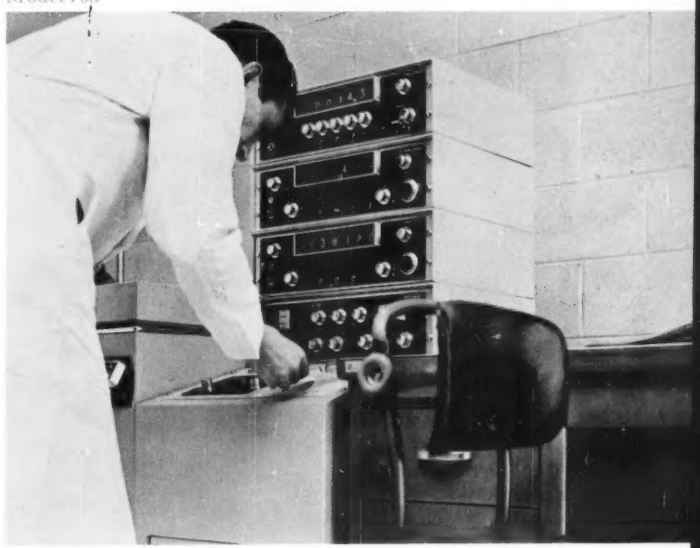
in ordinary room lighting. Output signals from the solid state circuitry are shaped pulses which can feed any standard scaling instrument. Nuclear-Chicago's Model 8250 Logic scaler is shown here mounted atop the 701 console.

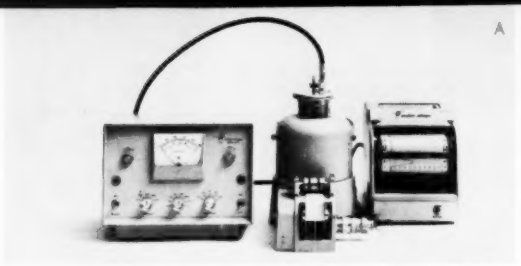
MODEL 702 has the dual-detector assembly mounted in a custom designed cooling chamber which permits highest efficiency for manual counting of C-14 and H-3 samples. The cooling chamber temperature is variable from $+10°$ to $+50°$F and is stable with $\pm 1°$F. A pushbutton control on the front of the cooling chamber operates the motor-driven elevator which positions the sample in the dual detector assembly. Logic 8250 scaler and Logic 8600 timer are shown.

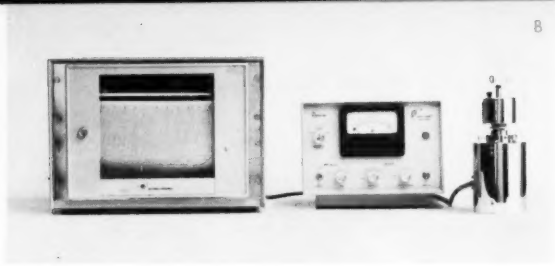
MODEL 703 is the most advanced counting system of its kind, providing both automatic sample changing and temperature controlled operation. Its sophisticated options of data presentation and versatile modes of automatic operation suggest it as the system of choice for laboratories counting many samples. A two-digit display on the cooling chamber shows the number of the sample which is in the counting position. The changer accepts up to 50 samples at a time, and an additional removable rack holds 50 more samples for temperature adaptation prior to measurement. Two 8250 scalers, 8600 timer, and 8401 digital recorder are shown.

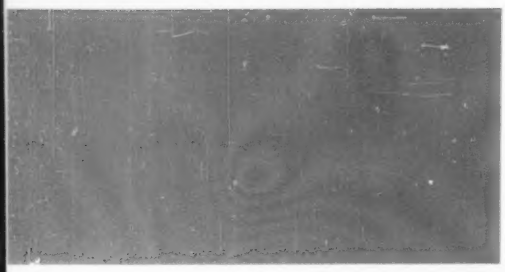
Model 701

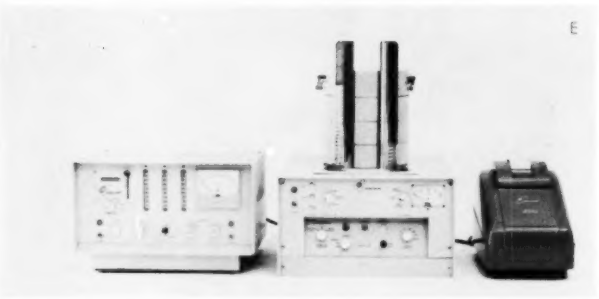
Model 703

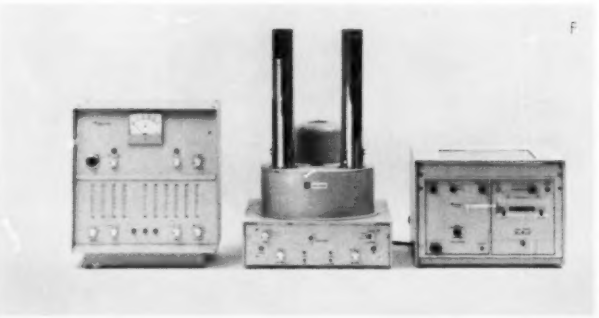












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E. C115 Automatic Beta Sample Changer is supplied with guarded gas-flow detector with background of only 2 cpm.

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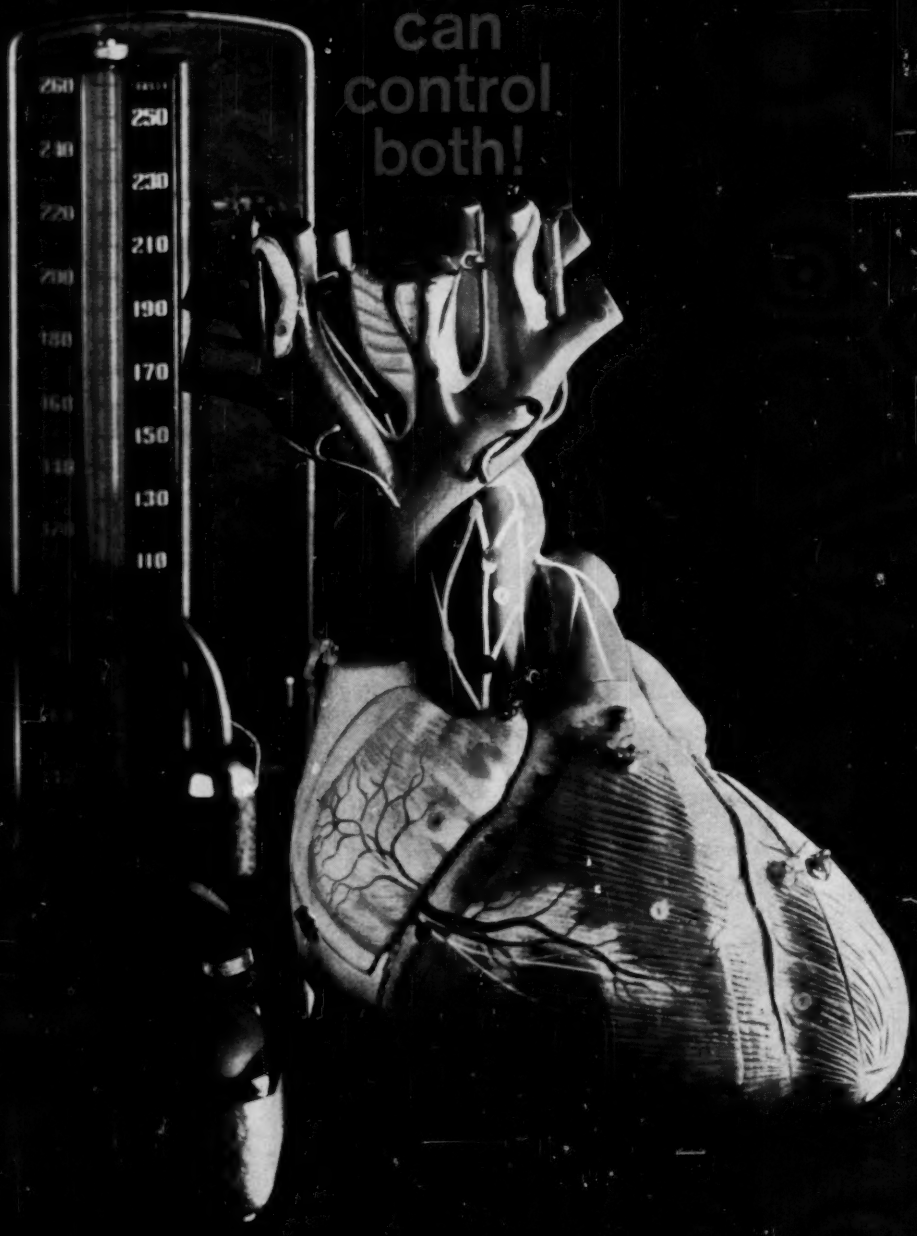


Serpasil lowers blood pressure gently, guards against cardiac damage

Serpasil—in addition to its well-established effectiveness in controlling high blood pressure—offers an important bonus in treating hypertension. Laboratory studies show that Serpasil can prevent stress-induced heart damage,[1,2] presumably through its ability to deplete the catecholamines (epinephrine and norepinephrine) from the myocardium.[3,4]

These laboratory data are clinically significant in light of growing evidence[5-7] that more than purely "mechanical" overwork may be involved in cardiac damage associated with hypertensive disease. Raab[5] suggests that much of this damage is due to a direct metabolic action of the catecholamines on heart muscle. The way to prevent it, he believes, is to deplete or inactivate excess catecholamines.

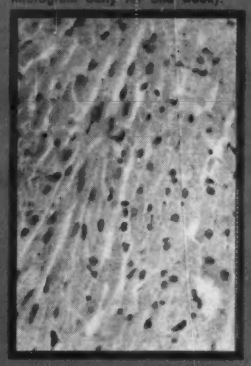
Thus, Serpasil not only eases the mechanical burden on the heart by reducing peripheral resistance and slowing heart rate, it may also provide protection against catecholamine-induced heart damage—the added benefit in prescribing Serpasil for hypertension.

References: 1. Raab, W., Stark, E., and Gigee, W. R.: Circulation 20:754 (Oct.) 1959. 2. Raab, W.: Research report to CIBA. 3. Carlsson, A., Rosengren, E., Bertler, A., and Nilsson, J.: Psychotropic Drugs (edited by Garattini, S., and Ghetti, V.), Elsevier Publishing Company, Amsterdam, 1957, pp. 363-372. 4. Waud, D. R., Kottegoda, S. R., and Krayer, O.: J. Pharmacol. & Exper. Therap. 124:340 (Dec.) 1958. 5. Raab, W.: Am. J. Cardiol. 5:971 (May) 1960. 6. Bayer, O., Borden, N. E., Bommighaus, H., and Effert, S.: Ztschr. klin. Med. 148:607 (June) 1950. 7. Raab, W.: Hormonal and Neurogenic Cardiovascular Disorders, The Williams & Wilkins Company, Baltimore, 1958, pp. 467, 468.

LABORATORY EVIDENCE SHOWS SERPASIL PREVENTS STRESS-INDUCED HEART DAMAGE

Severe heart damage in unprotected stressed rat. Tissue taken from rat given 2-α-methyl-9-α-fluorohydrocortisone and stressed (by restraint) for 15 hours. (Photomicrographs from Raab.[2])

No heart damage in stressed rat protected with Serpasil. Tissue taken from rat given 2-α-methyl-9-α-fluorohydrocortisone and stressed as at left, but also given Serpasil (0.4 microgram daily for one week).

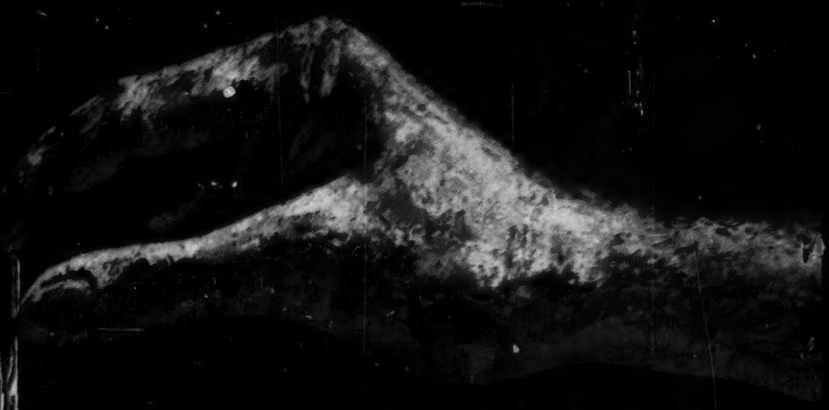


Note: While Serpasil did not completely protect the hearts of all animals in this study, it greatly reduced myocardial damage in most of them. Original magnification of photomicrographs: approximately 450 X.

Complete information about indications, dosage, cautions, and side effects of Serpasil—as well as a full report on its heart-protecting action—will be sent on request.

SUPPLIED: Tablets, 0.1 mg., 0.25 mg. (scored) and 1 mg. (scored).

Serpasil® CIBA Summit, N.J.



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THEORY OF THE USE OF ARTERIOVENOUS CONCENTRATION DIFFERENCES FOR MEASURING METABOLISM IN STEADY AND NON-STEADY STATES *

By KENNETH L. ZIERLER

(From the Department of Medicine, The Johns Hopkins University and Hospital, Baltimore, Md.)

(Submitted for publication July 14, 1961; accepted August 17, 1961)

In increasing numbers investigators are measuring concentrations of certain substances in arterial and in venous blood and equating arteriovenous (A-V) concentration differences obtained from simultaneous arterial and venous samples to uptake or release of the metabolite by a tissue drained by the venous system sampled. Conditions under which A-V concentration differences are valid estimates of tissue metabolism are sharply limited and, although the literature does contain ample warning (e.g., References 1–3), these limitations seem to have been misunderstood so often that it was thought a re-examination of the problem might be helfpul.

Fundamentally, all such investigations wish to employ the Fick principle (4) which states that if: *a*) arterial concentration of substance *x* is constant, *b*) if venous concentration of *x* is constant, *c*) if blood flow is constant and *d*) if uptake (or release) of *x* is constant, then, input of *x* (= flow multiplied by arterial concentration) = output of *x* (= flow multiplied by venous concentration) + tissue metabolism of *x* (where the tissue metabolism term is positive if there is tissue uptake and negative if there is tissue release).

Fick, of course, proposed this equation as a means of measuring blood flow. The equation can be and is used to find any single characteristic whenever all the others are measured independently, and it is our purpose to consider only its application to estimation of tissue metabolism.

It is obvious immediately that A-V concentration differences cannot be equated to tissue uptake (or release) unless flow is constant and known. It is correct to speak of changes in steady state A-V differences (i.e., the difference between one steady state and another steady state) as proportional to changes in tissue uptake (or release) if it is *known* that flow, although not quantified, has not changed. The curious practice has arisen of recognizing that flow must be included in the calculation but then ignoring it on the assumption that it is invariant. Those experienced in measurement of blood flow are familiar with its lability. Blood flow through the forearm of man may increase tenfold, for example, with exercise (5). When circulation to the hand is added to that of the forearm, flow through the brachial artery is easily doubled and, owing largely to the thermoregulatory function of blood flow to the hand, becomes unstable in the absence of careful control of ambient temperature and humidity. It is, therefore, not at all plausible to dismiss blood flow with the assumption that it was constant because the investigator was unaware that he had done anything to change it. Jiggling an arterial needle during sampling provokes vasodilatation, for example, so that most investigators who have not taken pains to lead blood from an artery through flexible tubing may have altered blood flow by as much as 50 per cent and perhaps even by more than twofold (observations by R. Andres, G. Cader and K. L. Zierler).

Because blood flow may not be constant we must inquire whether or not we can salvage application of the Fick principle by *a*) defining those variations in blood flow that are sufficiently small to permit use of the Fick principle (that is, if blood flow is treated as invariant, how much can it vary and produce no more than some given acceptable upper bound on the error estimate?), and *b*) redefining the Fick principle to include describable unsteady states of blood flow.

Validity of the use of the Fick principle depends not only on steady blood flow but also on constant arterial concentration and on constant tissue metabolism. It happens commonly that

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it is desired to measure tissue metabolism precisely when arterial concentration is changing or when metabolism may be changing. It is therefore equally necessary to ask how much change in arterial concentration or in metabolism is tolerable if the Fick principle is to be used or whether the Fick principle can be modified to include non-steady states with respect to arterial concentration or tissue metabolism.

Many investigators have stated or implied that even though, during non-steady states, an A-V difference may not measure accurately tissue uptake or release at that time, it must be true at least qualitatively that a positive A-V difference means that there is net tissue uptake, and a negative A-V difference means that there is net tissue release. This is utterly incorrect, as we shall show.

In substance, then, we ask what interpretation can be put on A-V differences during non-steady states.

Indicator dilution theory: tissue metabolism is zero

1. *Stationary systems.* The discussion which follows is based on principles developed for analysis of indicator dilution theory for measurement of blood flow and volume. Indicator dilution techniques rely on selection of a substance for which metabolism is zero; i.e., the Fick principle reduces to input = output for the steady state. The importance of indicator dilution theory for the present application is that the behavior of certain transient changes in input has been analyzed.

Consider a vascular bed with a single entrance and a single exit. Between entrance and exit, branching and intercommunication of blood vessels may be as complicated as you please. The exact form of the internal structure of the system is immaterial. Blood entering the system during some time interval, say between time t and $t + dt$, will not leave the system as a unit at some later time but will be dispersed with respect to time. The fraction of it which leaves the system s time units later between time $t + s$ and $t + s + ds$ is $h(s)\,ds$, where $h(s)$ is the fraction leaving per unit time; $h(s)$ is a distribution or frequency function with dimension 1/time. For example, if 20 per cent of blood which entered at time zero leaves the system per unit time during the fourth time interval after zero, then $h(4) = 0.2$. The sum of all $h(s)$ is unity; that is, sooner or later all the blood which entered at time t leaves the system and $\int_0^\infty h(t)\,dt = 1$.

The distribution function of transit times through the system is $h(s)$ or $h(t)$. If, for example, the shortest transit time is 3 time units and 30 per cent of the blood has such a transit time, 40 per cent of the blood has a transit time of 4 time units and the remaining 30 per cent of the blood has a transit time of 5 time units, then, $h(0) = 0$, $h(1) = 0$, $h(2) = 0$, $h(3) = 0.3$, $h(4) = 0.4$, $h(5) = 0.3$, $h(6) = 0$. The mean transit time, $\bar{t}$, is $(3 \times 0.3) + (4 \times 0.4) + (5 \times 0.3) = \int_0^\infty th(t)\,dt$.

An indicator is a substance which is so dispersed through the system that the distribution function of its transit times is the same as that of the native material under investigation. That is, the distribution function of transit times of the indicator, which we can observe and measure, gives us the desired distribution function of transit times of native unmarked material which we cannot otherwise observe.

If an indicator is introduced as some function of time, $i(t)$, into the entrance of the system, its concentration at exit is described by the relation which we shall develop provided the following conditions hold: *a*) flow through the system is constant; *b*) volume of the system is constant; *c*) indicator is mixed thoroughly with inflowing blood at the entrance to the system so that $i(t)/F = C_A(t)$, that is, injected indicator divided by appropriate fluid flow is the input concentration of indicator; *d*) all injected indicator leaves the system eventually, that is, there are no stagnant pools; *e*) the system is stationary, that is, the distribution function of transit times does not change during the period of observation. (This is a more strenuous limitation than demanding constant flow and volume, because it is possible to vary the distribution function and still hold flow and volume constant.)

We wish to describe concentration of indicator at exit at time t, $C_V(t)$, as a function of concentration of indicator at entrance, $C_A(t)$. Consider the contribution to the exit concentration at time t made by indicator which entered the

system s time units earlier, that is, at time $(t - s)$. The fraction of injected indicator leaving the system per unit time is $h(s)$. The amount of it leaving the system during the interval between s and $s + ds$ time units after introduction is $C_A(t - s) \cdot h(s) \cdot ds$. Now let s vary from 0 to t and sum $C_A(t - s) \cdot h(s) \cdot ds$ over all such time intervals. The sum is the desired concentration at exit, or

$$C_V(t) = \int_0^t C_A(t - s)\, h(s)\, ds \qquad [1]$$

Equation 1 is owing to Stephenson (6) and further discussion of it may be found in References 7–9. For present purposes we are interested in examining the effect on A-V differences of several functions that $C_A(t)$ might assume.

Consider first the behavior of Equation 1 when $C_A(t)$ is a constant $= I/F$ for $t \geq 0$ and $= 0$ for $t < 0$, where I is the constant rate of injection of indicator and F is constant blood flow. Equation 1 becomes

$$C_V(t) = \frac{I}{F} \int_0^t h(s)\, ds \qquad [2]$$

Because $\int_0^\infty h(t)\, dt = 1$, $\lim_{t \to \infty} C_V(t) = \frac{I}{F}$. $C_V(t)$ approaches that limit along the curve that is proportional to the cumulative frequency function of transit times, $\int_0^t h(s)\, ds = H(t)$. The cumulative frequency function of transit times describes the fraction of blood which has traversed the system by time t. Thus, if 30 per cent of the blood has a transit time of 3 time units, 40 per cent has a transit time of 4 time units and none has any shorter transit time, then at the end of the fourth time unit 70 per cent of the blood which entered at time zero has left the system, or $H(4) = 0.7$. The limit of $H(t)$, of course, is 1.

Depending on the exact nature of $h(t)$, $C_V(t)$ will look something like the curve drawn in Figure, 1. The A-V difference $C_A(t) - C_V(t) = (I/F) - C_V(t)$, is positive from time zero until some time at which the last particle of that indicator which was injected at zero time has left the system. At that instant $H(t) = 1$.

It is obvious, despite the fact that there is a period of time during which $C_V(t) < C_A(t)$, that there has been no tissue metabolism of indicator;

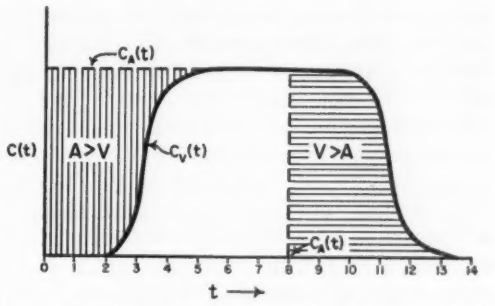

FIG. 1. INDICATOR DILUTION CURVE. Flow constant, and stationarity exists. Constant injection.

that is, a positive A-V difference does not necessarily mean that there has been uptake of the substance by tissues. In the case at hand, the area between the arterial and venous concentrations (see Figure 1) is exactly the mean transit time multipled by the steady state concentration of indicator, I/F (see Reference 7 for proof). The mean transit time, $\bar{t}$, is exactly the volume, V, in which indicator is distributed, divided by the flow, F, through the system. The integrated A-V difference is therefore proportional to V/F.

If at some time, T, after the steady state has been reached, injection of indicator ceases, arterial concentration at entrance falls immediately to zero. We now imagine that indicator is washed out of the system by indicator-free fluid and the equation becomes

$$C_V(t) = \begin{cases} 0, t < 0 \\ \frac{I}{F} \int_0^t h(s)\, ds, 0 \leq t \leq T \\ \frac{I}{F} \left(1 - \int_T^{t-T} h(s)\, ds\right), t \geq T \end{cases} \qquad [3]$$

Figure 1 demonstrates Equation 3. After time T, $C_V(t)$ exceeds $C_A(t)$ until all indicator is washed out of the system. This negative A-V difference, of course, does not mean that some tissue is producing the indicator and delivering it to the circulation. Indeed, the integrated negative A-V difference is exactly the same size as the integrated positive A-V difference and is a measure of $\bar{t}$.

There is nothing in this analysis which restricts the indicator to the vascular system. If one uses an indicator which diffuses into inter-

stitial space, so long as the original assumptions are met, one simply changes the distribution function of transit times to include extravascular paths. The flow remains the same. The volume measured is larger than that of the vascular system because it is the volume of distribution of indicator. The integrated A-V difference from time zero to T is therefore larger, but there is still no measure of tissue metabolism. The indicator may be distributed throughout tissue water. It will still obey Equation 3. The integrated A-V difference from time zero to T will be larger. It may even exceed the A-V difference of those metabolized substances which have an apparent volume of distribution less than that of total water.

Now let us consider the behavior of Equation 1 when $C_A(t)$ is not constant. A simple case lets $C_A(t)$ increase with constant velocity, that is,

$$C_A(t) = \begin{cases} 0, & t < 0 \\ a \cdot t, & t \geq 0 \end{cases}$$

where a is a constant. Equation 1 becomes

$$C_V(t) = a \int_0^t [t - s]\, h(s)\, ds$$

$$= at \int_0^t h(s)\, ds - a \int_0^t s h(s)\, ds \quad [4]$$

The asymptotic behavior of $C_V(t)$ is definite from Equation 4 because $\lim_{t\to\infty} \int_0^t h(s)\, ds = 1$ and $\lim_{t\to\infty} \int_0^t s\, h(s)\, d = \bar{t}$. Therefore,

$$\lim_{t\to\infty} C_V(t) = a \cdot (t - \bar{t}) = C_A(t - \bar{t})$$

that is, when all indicator introduced at time zero is washed out of the system, then $C_V(t)$ equals the arterial concentration introduced $\bar{t}$ time units earlier and $C_V(t)$ continues to be so related to $C_A(t)$ from then on. There will, therefore, always be a positive A-V difference even though there is obviously no tissue metabolism of indicator.

Now let $C_A(t)$ decrease linearly from some previously constant concentration; that is,

$$C_A(t) = \begin{cases} C_A(0), & t \leq 0 \\ C_A(0) - a \cdot t, & 0 \leq t \leq C_A(0)/a \\ 0, & t \geq C_A(0)/a \end{cases}$$

Then Equation 1 becomes

$$C_V(t) = C_A(0) - a \int_0^t [t - s]\, h(s)\, ds, \quad 0 \leq t \quad [5]$$

and the asymptotic behavior, until $C_A(t) = 0$ at $t = C_A(0)/a$, is

$$\lim_{t\to\infty} C_V(t) = C_A(t - \bar{t})$$

The A-V difference, once $C_V(t)$ reaches the above value, is $C_A(t) - C_V(t) = -a\,\bar{t}$; that is, the A-V difference is always negative.

It is instructive to combine a rising and falling arterial injection so that the envelope of arterial concentration is triangular with time, as in Figure 2. Then

$$C_A(t) = \begin{cases} 0, & t \leq 0 \\ a \cdot t, & 0 \leq t \leq T \\ a \cdot (2T - t), & T \leq t \leq 2T \\ 0, & t \geq 2T \end{cases}$$

Substitution of the appropriate values of $C_A(t - s)$ into Equation 1 yields the curve of $C_V(t)$ for $a = 1$ and for the function $h(t)$ illustrated in Figure 2. The area under the curve $C_A(t)$ equals the area under the curve $C_V(t)$ because all injected indicator must leave the system. $F \int_0^\infty C_A(t)\, dt$ is the total indicator injected and $F \int_0^\infty C_V(t)\, dt$ is the total indicator leaving the system. It follows immediately, therefore, that the shaded area marked $A > V$ in Figure 2 equals the shaded area marked $V > A$. The points to

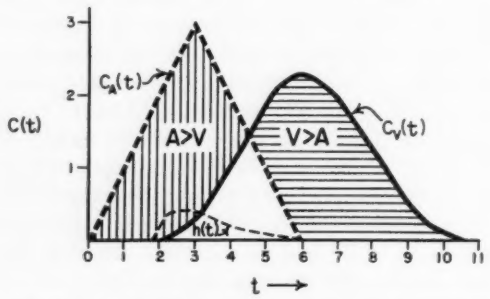

FIG. 2. INDICATOR DILUTION CURVE. Flow constant, and stationarity exists. $C_A(t)$ increases linearly, then decreases linearly.

be noted in Figure 2 are: that the maximal venous concentration is less than the maximal arterial; that until 4.5 time units $C_A > C_V$, although there is no tissue uptake, and after 4.5 time units $C_V > C_A$, although there is no tissue production; and that C_V continues to rise for a time after C_A has begun to fall.

We can now make a general statement about A-V differences of indicators during transient changes in indicator input effected by altered $C_A(t)$. Assume that $C_A(t)$ and $C_V(t)$ are constant for a sufficiently long time before $t = 0$ and that at $t = 0$ a change in $C_A(t)$ begins. Equation 1 can be integrated by parts to yield

$$C_V(t) = \int_0^t C_A(t - s)\, h(s)\, ds$$
$$= C_A(t - s)\, H(s)\,|_0^t + \int_0^t C'_A(t - s)\, H(s)\, ds \quad [6]$$

where $H(t)$ is the cumulative distribution function $\int_0^t h(s)\, ds$, C'_A is the first derivative of C_A and $d\ C_A(t)/dt = -\ d\ C_A(t - s)/ds$.

Because $H(0) = 0$ in the real vascular system, $C_A(t - s)\ \ H(s)\,|_0^t = C_A(0)H(t)$.

Because $H(t) \leq 1$, if $C'_A(t) > 0$ (that is, if $C_A(t)$ is increasing with time), $\int_0^t C'_A(t - s)\ H(s)\ ds < \int_0^t C'_A(t - s)\ ds = C_A(t) - C_A(0)$.

Substituting these functions in the integration by parts yields $C_V(t) < C_A(0)H(t) + C_A(t) - C_A(0) = C_A(t) - C_A(0)\ [1 - H(t)]$, or $C_V(t) < C_A(t)$.

If arterial concentration is decreasing with time, $C'_A(t) < 0$ and $\int_0^t C'_A(t - s)\ H(s)\ ds > \int_0^t C'_A(t - s)\ ds$. With this substitution and by argument similar to that used above, $C_V(t) > C_A(t)$.

Thus, if arterial concentration increases there is a positive A-V difference. If arterial concentration decreases there is a negative A-V difference. If arterial concentration is constant for a sufficiently long period (that is, long enough for $H(t)$ to become unity) then venous concentration will, when $H(t) = 1$, equal arterial concentration. A-V differences occur because positive times are required for traversal of the vascular net (that is, because $H(0) \neq 1$). Clearly, then, A-V differences during non-steady states are not in themselves evidence of tissue metabolism.

2. *Nonstationary systems; variable flow.* We have so far been concerned with input changes produced by altered concentration at constant flow. Now consider input changes produced by altered flow.

Consider first the simplest but least likely case, in which flow is altered without altering the distribution of transit times (that is, stationarity exists). This is possible if the relation $V/F = \bar{t}$ through every path is constant. Such an event is handled simply by generalizing Equation 1

$$m_0(t) = \int_0^t m_i(t - s)\, h(s)\, ds \quad [7]$$

where $m_0(t)$ is output (= flow ... time t multiplied by venous concentration at time t) at time t and $m_i(t)$ is input at time t. If $F_0(t)$ is flow at output, $F_i(t)$ is inflow, and C_A is the constant arterial concentration, then Equation 7 becomes

$$C_V(t) = \frac{C_A}{F_0(t)} \int_0^t F_i(t - s)\, h(s)\, ds.$$

Since nothing has been done to alter concentration anywhere in the system, it must be that $C_V(t) = C_A$. Therefore,

$$F_0(t) = \int_0^t F_i(t - s)\, h(s)\, ds \quad [8]$$

In a system of rigid tubes containing incompressible fluid such as water, flow everywhere in the system is identical at any time. In particular, $F_0(t) = F_i(t)$, the limiting case in which $h(t)$ is simply a brief spike beginning at $t = 0$.

More realistically we must consider that a change in flow is associated with a change in the distribution of transit times through the system, that is, there is nonstationarity. Then if flow, F, is a function of time, $F(t)$, so is the distribution of transit times a function, not only of elapsed time following introduction of indicator, but also of absolute time at which indicator was introduced. The problem has been explored by Sherman (10) following methods used by Zadeh

(11) in analysis of variable or dynamic electrical networks which depend on specified combinations of input, output and time. The following development relies largely on the work of Zadeh and of Sherman.

We have until now defined the time, or the center of gravity of the time interval at which indicator is added to the system, as zero time. Let us now permit this time of indicator addition to be any time, x. Then $h(x)$ is the distribution function of transit times through the system which exists at time x. But the distribution function of transit times is also a function of the time elapsed since addition of indicator, a time which we will designate a, where $a = t - x$, the difference between absolute time, t, and time of indicator addition. The distribution function is therefore $h(x, a) = h(x, t - x) = h(t - a, a)$.

Because $h(x, a)$ is a distribution function (that is, because all indicator introduced at any given time x must eventually leave the system)

$$\int_x^\infty h(x, t - x) \quad dt = \int_x^\infty h(t - a, a) \quad dt = 1$$

Now consider that $h(x, a)$ is the distribution function not only of indicator particles but of all fluid that entered the system at any given time x.

Consider the contribution to outflow at time t made by fluid that entered the system at a particular time before t, x_1. Of the total inflow $F_i(x_1)$, the fraction leaving at a time units later [that is, between times t and $t + dt$ $(= x_1 + a$ and $x_1 + a + da)]$ is $F_i(x_1)\ h(x_1,\ a)\ da$, or $d\ F_0(t) = F_i(x_1)\ h(x_1, a)\ da$. The total outflow is obtained simply by integrating the above expression for $d\ F_0(t)$, letting x vary over all previous times before t, or

$$F_0(t) = \int_{-\infty}^{t} F_i(x)\ h(x, t - x)\ dx \qquad [9]$$

It is implicit in Equation 9 that once a particle enters the system at any given time x it is committed to traverse the system at some given transit time a with the probability defined by $h(x, a)$, and that changes in flow occurring at some other x, and therefore changes in $h(x, a)$ do not affect transit times of particles already in the system. Further, as Sherman pointed out (10), $F_0(t)$ is defined only in terms of $F_i(x)$ and not in terms of $F_i(t)$, the simultaneous inflow.

By an argument analogous to that used to develop Equation 9, output of indicator as a function of input and time is

$$F_0(t)\ C_V(t) = \int_{-\infty}^{t} F_i(x)\ C_A(x)\ h(x, t - x)\ dx \qquad [10]$$

For the case of constant arterial concentration C_A, venous concentration at time t is

$$C_V(t) = \frac{C_A}{F_0(t)} \int_{-\infty}^{t} F_i(x)\ h(x, t - x)\ dx = C_A$$

which is expected because C_A is neither diluted nor concentrated as it passes through the system.

For the special case in which $F_i(x)$ and $C_A(x)$ vary inversely so that their product is constant, I, Equation 10 becomes

$$C_V(t) = \frac{I}{F_0(t)} \int_{-\infty}^{t} h(x, t - x)\ dx \qquad [11]$$

After sufficiently long time, because the integral in Equation 11 becomes unity, limit of $C_V(t) = I/F_0(t)$. Until that time is reached, there will be an A-V concentration difference.

We can define precisely what is meant by a sufficiently long time with the aid of Figure 3. Several possible distribution functions are illustrated as histograms by A, C, and F. The total area of each distribution function is unity. Experimentally, these distribution functions are determined by injecting quickly a single quantity of indicator into the entrance to the system under study and measuring its concentration at exit during a period of stationarity. In communication engineering this is equivalent to applying a brief pulse to the input to a network and observing the response at output. In B the unit pulse is applied repeatedly, as indicated by the arrows, or in a hydrodynamic system indicator is injected at constant rate. Output distribution functions are superposed until the height of the output response becomes unity, and it remains unity as long as constant input is maintained. Although when output response is unity it equals the unit input, the output response does not represent any single unit input admitted at some earlier time but it is made up of contributions from a number of earlier inputs. The time at which the output response reaches unity is exactly the longest transit time in the system,

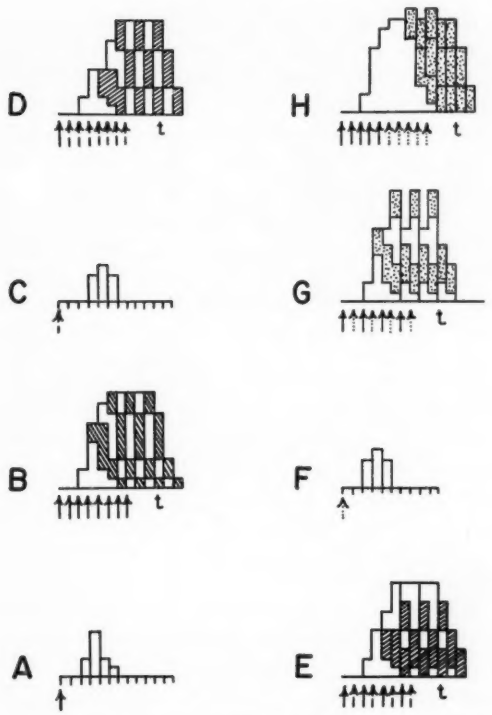

FIG. 3. VARIOUS DISTRIBUTION FUNCTIONS AND THEIR COMBINATIONS IN STATIONARY AND NONSTATIONARY STATES. Alternating white and shaded areas represent distribution of materials entering at successive time intervals. Each arrow style represents a different distribution function pertinent to entrance time indicated by arrow. The functions are: A. $h(x, a) = h_1(t)$. B. $\int_0^t h(s)\, ds = \int_{x_1}^t h(x, t - x)\, dx$, where $h(s) = h_1(t)$. C. $h(x, a) = h_2(t)$. D. $\int_{x_1}^t h(x, t - x)\, dx$, where $h(x_1) = h_1(t)$, $h(x_2)$ to $h(x_8) = h_2(t)$. E. $\int_{x_1}^t h(x, t - x)\, dx$, where $h(x_1) = h(x_3) = h(x_5) = h(x_7) = h_1(t)$ and $h(x_2) = h(x_4) = h(x_6) = h(x_8) = h_2(t)$. F. $h(x, a) = h_3(t)$. G. $\int_{x_1}^t h(x, t-x)\, dx$, where $h_1(t)$ and $h_3(t)$ exist at alternate time units. H. $\int_{x_1}^t h(x, t - x)\, dx$, where $h(x_1)$ to $h(x_5) = h_1(t)$, $h(x_6)$ to $h(x_{10}) = h_3(t)$.

determined from the single sudden injection curve A.

In Figure 3D, constant injection of indicator occurs but the system is nonstationary in that the distribution function changes from $h_1(t)$ during the first time interval to $h_2(t)$ for all subsequent time intervals. Output, which is superposed distribution functions, reaches unity at a time t exactly equal to x_2, the time at which the distribution function through the system changed from h_1 to h_2, plus the longest transit time in the function $h_2(t)$ or in $h_1(t)$, whichever is longer.

In Figure 3E and G there is constant injection through a nonstationary system. In both cases the distribution function alternates between two functions. In E, the longest transit time of both functions is the same. The output reaches unity exactly at that longest transit time and remains there. In G, the longest transit time of one distribution function differs from that of the other. The output response eventually (at a time equal to the greater of the longest transit times of the two distribution functions) oscillates symmetrically about unity, exceeding unity by as much as it is alternately less than unity. The frequency of output oscillation is exactly that at which the two distribution functions alternate.

In Figure 3H, the system changes from one steady state, in which the distribution function is $h_1(t)$ to a second state in which the distribution function is $h_2(t)$. The time required for output to restabilize at unity after the change is made is exactly the longest transit time in the new distribution function.

To return to Equation 11, the sufficiently long time required for $C_V(t)$ to equal $I/F_0(t)$ is either the longest transit time of any distribution function which describes the system for a time at least equal to its longest time or, if the system is always nonstationary but oscillates in some regular way about a group of distribution functions, it is the longest of all transit times described by any distribution function and $C_V(t)$ can be taken as a weighted mean of its oscillating value.

The relation between flow and the distribution function may be confusing. It is possible, but not likely, that a change in the distribution of transit times could occur without a change in flow and it has already been stated that a change in flow could occur without a change in distribution function if volume changed appropriately, but this is again unlikely. Therefore, unless it can be demonstrated otherwise in a specific case, whenever flow changes it is safer to assume that the distribution function also changes and Equation 10 is more apt to be descriptive of the real system than is Equation 7.

But the basic assumption underlying Equa-

tion 10 is probably an oversimplification so that even Equation 10 may be inadequate. The assumption that distribution functions are superposable rests formally on the assumption that the system is linear; that is, that the relation between input and output can be described by a linear equation. This assumption implies that once a particle has entered the system it is committed to traverse it in a given transit time; that is, changes in the distribution function of transit times are assumed not to affect particles already in the system but only new particles just entering the system.

This assumption is not apt to be valid in general. When the distribution function changes owing to vasoactivity, for example, the transit times of particles already in the system are probably changed. In the extreme, with closure of some portions of a capillary bed, flow ceases and diffusion, which until now has been considered negligible, becomes the only means by which solute particles can move through the system; that is, movement of solute and solvent, previously considered to be coupled and described by a common distribution function, becomes uncoupled so that one distribution function does not describe transit times through the system. The system is, therefore, probably formally nonlinear and the development of Equation 10, which rests on the superposition principle, is inappropriate. There is no simple formal solution to this problem. A description of the relations between input, output and time in such a nonlinear system requires detailed knowledge of the behavior of every particle within the system as a function of position within the system and time, and there is no experimental method for obtaining the necessary information at present. This means that in nonstationary and nonlinear systems there is no way to relate simultaneous arterial and venous concentrations, and no simple correction factor, such as evolved from discussion of Equation 4, is applicable.

Tissue metabolism not zero

1. *Tissue uptake.* We have been examining in increasing complexity A-V concentrations of substances for which tissue uptake or output is zero. The problem is even more difficult when we let tissue uptake assume nonzero values.

In the first place, the distribution of transit times of a substance, some of which is metabolized, is different from the distribution of transit times of a solute confined to plasma or to total extracellular fluid and is even apt to be different from that of total water. The transit times under consideration are only of those particles which leave the system and so contribute to the venous concentration. If tissue uptake from every channel through the system were proportional to the rate at which the metabolizable substance flowed through that channel, then and only then, would the distribution of transit times of the substance be identical with the distribution of transit times of some nonmetabolized indicator. Although there may be in certain special cases a close relation between vascularity and metabolism, in general, we anticipate that the fractional uptake from some channels will be different from that in others. There might be little uptake from blood through channels with very short transit times, the extreme being the case of arteriovenous shunts, and there might be a great deal of uptake from blood coursing through intricate capillary nets with long transit times.

For example, if $h(t)$, the distribution function of indicator transit times is $h(0) = 0$, $h(1) = 0$, $h(2) = 0.3$, $h(3) = 0.4$, $h(4) = 0.3$, $h(5)$, and so forth $= 0$, then the distribution function of transit times, $g(t)$, of a metabolized substance through the same vascular bed might be $g(0) = 0$, $g(1) = 0$, $g(2) = 0.5$, $g(3) = 0.3$, $g(4) = 0.2$, $g(5)$, and so forth $= 0$. In this example, the mean transit time for nonmetabolized particles, $\int_0^\infty t\,h(t)\,dt$, is longer than the mean transit time for metabolized particles, $\int_0^\infty t\,g(t)\,dt$, because a large fraction of metabolized particles coursing through long-transit-time channels was eliminated by metabolism within the system.

The purpose of this example is to make it clear that no simple known relation exists between transit times of indicators and of metabolities. It is therefore not possible to "correct" observed venous concentrations by displacement along the time axis in accordance with some notion of the distribution of transit times of nonmetabo-

lized indicators, although the correction can be made as a reasonable approximation in those cases in which nearly all of the substance traverses the system; that is, in those cases in which only a small fraction of entering material is taken up by tissues. For example, in the forearm of man at rest in the basal state about 98 per cent of glucose delivered by arterial blood appears in forearm venous effluent (12). In this example, then, $g(t)$ is probably very nearly the same as $h(t)$ so that for certain practical purposes it would be acceptable to assume that $g(t)$ was indeed $h(t)$.

The relation between $h(t)$ and $g(t)$ is easily defined. Consider those particles whose transit times are between t and $t + dt$ time units. Of those nonmetabolizable or indicator particles entering at zero time, the fraction leaving in the time interval t to $t + dt$ is $h(t)\ dt$. Of metabolizable particles, the fraction of those that entered at zero time which leave in the same time interval is $r(t)\ h(t)\ \ dt$, where $0 \leq r(t) \leq 1$. Integration over all time yields the fraction of metabolizable particles which entered at zero time and which eventually leave the system, $p = \int_0^\infty r(t)\ h(t)\ dt$ and $0 \leq p \leq 1$. Therefore, of those metabolizable particles entering at zero time and which are not metabolized, the fraction leaving the system per unit time between t and $t + dt$ time units later is

$$g(t) = r(t)\ h(t)/p \qquad [12]$$

When $g(t) = h(t)$, from Equation 12 $r(t) = p$, a constant, or $g(t)$ can equal $h(t)$ only if $r(t)$ is constant. When p is almost unity, there can be no assurance that any given $g(t) \doteq h(t)$, but $\int_0^t C_A(t - s)\ \ g(s)\ \ ds \doteq \int_0^t C_A(t - s)\ \ h(s)\ \ ds$ for sufficiently large t.

One can, during steady states, observe the distribution function of transit times of certain metabolizable substances by using tracer quantities of labeled material otherwise identical with the metabolized substance. This distribution function, $g(t)$, can be evaluated only while there is stationarity. This requires not only that flow and volume remain constant but also that there be constant uptake of metabolite per unit time, $\dot{Q}$, for if rate of uptake changes with time it cannot be assumed that the proportional change in every channel is identical, which is required if $g(t)$ is to be the same.

The quantity of metabolite entering the system between time t and $t + dt$ is $F\ C_A(t)\ dt$. Of this quantity, assuming uptake is constant, the total amount leaving the system during all subsequent time intervals is $[F\ C_A(t) - \dot{Q}]\ dt$. By the arguments used to develop Equation 1

$$C_V(t) = \int_0^t \left[C_A(t - s) - \frac{\dot{Q}}{F} \right] g(s)\ ds$$

$$= \int_0^t C_A(t - s)\ g(s)\ ds - \frac{\dot{Q}}{F} G(t) \qquad [13]$$

where $G(t) = \int_0^t g(s)\ ds$, and $F\ C_A(t) \geq \dot{Q}$.

In a form analogous to that of the classical Fick equation, Equation 13 becomes

$$\dot{Q} = \frac{F}{G(t)} \left[\int_0^t C_A(t - s)\ g(s)\ ds - C_V(t) \right]$$

In the discussion of Equation 6 we showed that if $d\ C_A(t)/dt > 0$, $\int_0^t C_A(t - s)\ g(s)\ ds < C_A(t)$, and if $d\ C_A(t)/dt < 0$, $\int_0^t C_A(t - s)\ \ g(s)\ \ ds > C_A(t)$. Therefore, after sufficient time has elapsed so that $G(t)$ has passed to unity, if $C_A(t)$ is increasing, the A-V difference overestimates $\dot{Q}$, and if $C_A(t)$ is decreasing the A-V difference underestimates $\dot{Q}$. Indeed it is even possible for the A-V difference to be negative, if $d\ C_A(t)/dt$ is sufficiently negative and the mean transit time is sufficiently long, despite the fact that there may be real tissue uptake of the substance measured.

Except where $g(t) = h(t)$, it is not possible to use an indicator curve, such as that in Figure 2, as a guide in estimation of $\dot{Q}$ during transient changes in $C_A(t)$. For example, if in Figure 2 $C_A(t)$ were displaced upward, as in Figure 4, by some constant for all t so that there was a constant steady state A-V difference, the A-V difference during the transient would not be that of Equation 12 unless the distribution function, $h(t)$, of the indicator happened to be the same as that of the metabolite, $g(t)$. If $g(t)$ can be determined independently and can be proven

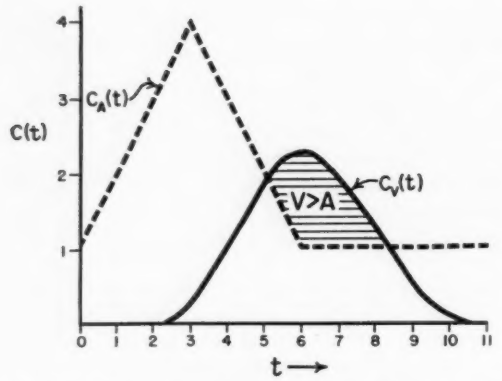

FIG. 4. CONSTANT UPTAKE OF A SUBSTANCE WITH SAME CHANGES IN ARTERIAL CONCENTRATION AS IN FIGURE 2. Uptake cannot be calculated accurately at any time during changes in arterial or venous concentration. Flow multiplied by integrated A-V difference from time 0 to 11 is, in this example, total tissue uptake over that time interval.

stationary, then Equation 13 can be used to test whether or not $\dot{Q}$ is in fact constant.

If tissue uptake is not constant but is some function of time, $\dot{Q}(t)$, then we must consider that the system is not stationary. Consider first the more general problem of varying flow and varying uptake.

If we assume that the system is linear—that is, that the distributions of inputs are superposable—as we did in development of Equation 10 when we considered effects of altered flow and distribution function on indicator concentration, we can immediately generalize Equation 10 by analogy

$$m_0(t) = \int_{-\infty}^{t} p(x)\, m_i(x)\, g(x, t - x)\, dx \quad [14]$$

where $m_0(t) = F_0(t)\ C_V(t)$, $m_i(t) = F_i(t)\ C_A(t)$ and $p(t)$ is that fraction of $m_i(t)$ which is not metabolized (see Equation 12). For constant uptake, Equation 14 becomes

$$m_0(t) = \int_{-\infty}^{t} [m_i(x) - \dot{Q}]\, g(x, t - x)\, dx \quad [15]$$

and for constant arterial concentration, Equation 15 becomes

$$C_V(t) = \frac{C_A}{F_0(t)} \int_{-\infty}^{t} F_i(x)\, g(x, t - x)\, dx - \frac{\dot{Q}}{F_0(t)} \int_{-\infty}^{t} g(x, t - x)\, dx \quad [16]$$

Unlike the case of indicator dilution at constant arterial concentration and varying flow, in which $C_A(t) = C_A$, a constant (see discussion following Equation 10), when there is tissue uptake a change in flow produces a change in venous concentration even if arterial concentration is held constant. It is intuitively evident that this must also be true in nonlinear systems. Figure 5 illustrates the sort of changes one might find when flow is changed in stepwise fashion so that the system moves from one steady state to another. An increase in inflow, C_A and $\dot{Q}$ remaining constant, produces a transient change in outflow and in venous concentration, which lag behind input changes. Owing to the lag there is a transient increase in A-V difference and $\dot{Q}$ is overestimated. The steady state is reached at a time equal to the longest transit time of metabolizable particles through the system, not the longest transit time of plasma. The overestimate in $\dot{Q}$ is not compensated in any way by a subsequent reduction in A-V difference as long

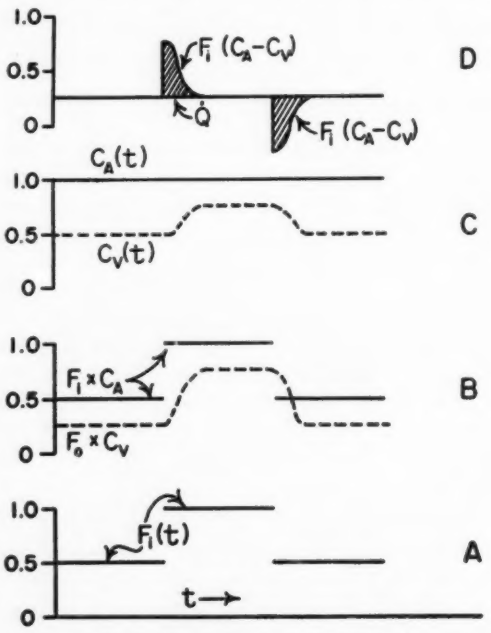

FIG. 5. EFFECT OF CHANGE IN FLOW ON A–V DIFFERENCE. A. Step increase in flow. B. Step increase in input with consequent change in output. C. Change in output concentration with change in flow, despite constant arterial concentration and constant uptake. D. Although tissue uptake, $\dot{Q}$, is constant, the product of flow and A-V difference is not constant when there is a step change in flow.

as the new steady state is maintained. This does not mean that there is tissue storage of the apparent excess, but that there is always a lag between input and output so that $F_0(t)\ C_V(t)$ is not made up of those particles that entered at t, but of those that entered over some time period before t. When flow is decreased to the original rate, there is a transient decrease in A-V difference and $\dot{Q}$ is underestimated. Indeed, if the decrease in flow is sufficiently great there may even be negative A-V differences, as in the illustration, and it may be concluded erroneously that there is tissue output. If, when flow returns to the original rate, the distribution of transit times also returns to the original distribution, the overestimate of $\dot{Q}$ which occurred when flow increased is exactly the same as the underestimate of $\dot{Q}$ which occurred when flow decreased. The integrated A-V difference multiplied by flow during the entire time interval will therefore measure total uptake over that time interval.

Although the assumption of linearity is not apt to hold when the system is nonstationary owing to altered flow, for reasons discussed earlier, the system may very well remain linear when nonstationarity is owing to altered metabolism in the presence of constant flow. This greatly simplifies the problem, as follows.

For the case of constant flow Equation 14 becomes

$$C_V(t) = \int_{-\infty}^{t} p(x)\ C_A(x)\ g(x, t - x)\ dx \quad [17]$$

Equation 17 does not tell us what tissue uptake was at time t. Although the quantity of material entering between time t and $t + dt$ which is metabolized eventually is $(1 - p)\ F\ C_A(t)\ dt$, this quantity is not $\dot{Q}\ dt$ because there is a distribution of transit times from entrance to the various sites of metabolism within the system. By analogy with equations developed previously in this paper for nonstationary linear systems, rate of tissue uptake as a function of time

$$\begin{aligned}\dot{Q}(t) &= F \int_{-\infty}^{t} [1 - p(x)]\ C_A(x)\ m(x, t - x)\ dx \\ &= F \int_{-\infty}^{t} C_A(x)\ m(x, t - x)\ dx \\ &\quad - F \int_{-\infty}^{t} p(x)\ C_A(x)\ m(x, t - x)\ dx \quad [18]\end{aligned}$$

where $m(x, a)$ is the distribution function of transit times from entrance to sites of uptake.

The integral in the second term on the right-hand side of Equation 18 is obviously related to the integral of Equation 17 which describes $C_V(t)$, the difference being in the distribution functions. The integral in the first term of Equation 18 is clearly related to $C_A(t)$, but it is also clearly not $C_A(t)$. Equation 18, in short, is a general form of the classical Fick equation for constant flow but for variable arterial concentration and variable uptake.

When $C_A(t)$ is constant, C_A, Equation 18 becomes

$$\begin{aligned}\dot{Q}(t) &= F\ C_A \int_{-\infty}^{t} m(x, t - x)\ dx \\ &\quad - F\ C_A \int_{-\infty}^{t} p(x)\ m(x, t - x)\ dx \quad [19]\end{aligned}$$

After a sufficiently long time, the first term in Equation 19 becomes $F\ C_A$. If $p(t)$ is also constant Equation 19 becomes $\dot{Q}(t) = F\ C_A(1 - p) \int_{-\infty}^{t} m(x, t - x)\ dx$, and for t sufficiently large, limit $\dot{Q}(t) = F\,C_A(1 - p) = F\,(C_A - C_V)$, which is the classical Fick equation.

Equation 18 is unfortunately not very useful in that form because it contains the distribution function, $m(x, a)$, which is not subject to direct experimental investigation. An approximate evaluation of $m(x, a)$ comes from the following analysis.

Consider what would have been the distribution of transit times, $f(t)$, through the system of only those particles which were removed. That is, if their transit times had not terminated within the system as described by $m(t)$, what would have been their transit times? Refer to the argument by which Equation 12, relating $g(t)$ to $h(t)$, was developed. Clearly,

$$f(t) = \frac{[1 - r(t)]\ h(t)}{1 - p} = \frac{h(t) - p\ g(t)}{1 - p}$$

Some relation must exist between the transit time of a particle to the point of uptake and its transit time through the entire system. It is plausible to assume that points of uptake lie approximately midway in time between entrance and exit. On this assumption, $m(2t) = f(t)$,

and Equation 18 becomes

$$\dot{Q}(t) \doteq F \int_{-\infty}^{t} C_A(x)\, h\left(\frac{t-x}{2}\right) dx - F \int_{-\infty}^{t} C_A(x)\, p(x)\, g\left(x, \frac{t-x}{2}\right) dx \quad [20]$$

Equation 20 is much simpler to handle than Equation 18. The first term on the right-hand side deals only with a stationary system, that is, as long as flow is constant (and volume), the distribution of transit times of the appropriate indicator, described by $h(t)$, is not dependent on time. We have already seen how to handle such a function when we considered indicator dilution through stationary systems. The second integral on the right-hand side of Equation 20 is closely related to $C_V(t)$, defined in Equation 17. Indeed, if the system becomes stationary for a sufficiently long time (the longest transit time) the second term on the right-hand side of Equation 20 equals $F\, C_V(t)$.

From the discussion surrounding Figure 2 and Equations 4–6, after sufficiently long time, for the case of linear increase in $C_A(t)$, which is a useful case because many changes in arterial concentration may be considered linear over some practicable time interval,

$$\dot{Q}(t) \doteq F\, C_A(t - \tfrac{1}{2}\,\bar{t}) - F\, C_V(t + \tfrac{1}{2}\,\bar{t}_g) \quad [21]$$

where $\bar{t}$ is mean time through $h(t)$ and $\bar{t}_g$ is mean time through $g(t)$. For the special case of $\dot{Q}(t) = 0$, $\bar{t} = \bar{t}_g$ and Equation 21 reduces to the limiting indicator dilution equation, $C_V(t) = C_A(t - \bar{t})$.

In short, if $C_A(t)$ increases linearly an approximate measure of $\dot{Q}(t)$ can be obtained by moving back in time $\frac{1}{2}\,\bar{t}$ units and noting C_A, by moving up in time $\frac{1}{2}\,\bar{t}_g$ units and noting C_V. The difference between these two corrected concentrations is nearly equal to $\dot{Q}(t)/F$; $\bar{t}_g$ can be obtained by tracer techniques or, for the case of substances such as glucose traversing the forearm of man, in which about 98 per cent of entering material leaves the system, $\bar{t}_g$ may be represented satisfactorily by $\bar{t}$.

From the discussion surrounding Figure 1 and Equations 2 and 3 for the case of constant arterial concentration and relatively linear changes in uptake, after sufficiently long time

$$\dot{Q}(t) \doteq F\, C_A - F\, C_V(t + \tfrac{1}{2}\,\bar{t}_g) \quad [22]$$

For the special case of $\dot{Q}(t) = 0$, $\frac{1}{2}\,\bar{t}_g$ degenerates to zero because it appears in Equation 22 only as an approximation of $\bar{t}_m$ which, of course, is nonexistent if there is no tissue uptake. Equation 22 states that even if input to the system (flow and arterial concentration) is constant, $\dot{Q}(t)$ is not equal to $F\, C_A - F\, C_V(t)$, but venous concentration must be corrected backward in time by an amount assumed to represent mean time from entrance to points of uptake.

2. *Tissue release.* We have so far spoken only of uptake and not of output of metabolites. In a steady state, the excess of the constant venous concentration above constant arterial concentration represents the ratio of constant tissue output to constant flow. In non-steady states there is no such symmetry between the analysis of uptake and of output. Consider the case in which a substance is introduced into our test system by way of arterial inflow and all of it eventually leaves the system; that is, there is no uptake. The test tissue, however, releases additional quantities of the substance. The concentration at output is therefore

$$C_V(t) = \int_0^t C_A(t - s)\, h(s)\, ds + \frac{1}{F} \int_0^t \dot{q}(t - s)\, n(s)\, ds \quad [23]$$

where $\dot{q}(t)$ is rate of tissue release at time t, and $n(t)$ is the distribution function of transit times from points of release from tissue cells to the exit from the system.

If a change in $\dot{q}$ is also associated with a change in $n(t)$, that is, if the distribution of released material is not stationary, and we must assume that this is apt to be the case, it is quite likely that as long as $h(t)$ describes a stationary system, the distribution of released material can be described by a linear net. This means simply that although the sites of and relative contributions to tissue release may vary with time,once the metabolite is released it is distributed through elements of the system described by $h(t)$, which is a stationary system. By analogy with previous discussions of linear nonstationary systems, Equation 23 is immediately generalized to

$$C_V(t) = \int_0^t C_A(t - s)\, h(s)\, ds + \frac{1}{F} \int_{-\infty}^{t} \dot{q}(x)\, n(x, t - x)\, dx \quad [24]$$

The first term on the right-hand side of Equation 24 is simply an indicator dilution term and obviously Equation 24 reduces to the indicator dilution Equation 1 for $\dot{q}(t) = 0$. This means that all of the discussion of effect of varying arterial concentration on indicator dilution curves is applicable to Equation 24. The second term is not the rate at which tissues release the substance at time t, but simply the sum of all contributions made by tissue release before time t which reach the sampling site at exit at time t.

For the case of $C_A(t)$ constant, Equation 24 becomes

$$F[C_V(t) - C_A] = \int_{-\infty}^{t} \dot{q}(x)\, n(x, t - x)\, dx \quad [25]$$

that is, flow multiplied by simultaneous A-V difference is not tissue release at time t but is the sum of those fractions of tissue release at time x whose transit times are $(t - x)$.

If $\dot{q}(t)$ increases linearly, assuming that the mean transit time from sites of release to exit is half the mean transit time from entrance to exit, and if $C_A(t)$ is constant, $F[C_V(t) - C_A] \doteq \dot{q}(t - \frac{1}{2}\bar{t})$, that is, use of simultaneous A-V difference determines the rate of tissue release which occurred approximately $\frac{1}{2}\bar{t}$ time units earlier.

If $\dot{q}(t)$ is constant but $C_A(t)$ is variable, $F C_V(t) - F \int_0^t C_A(t - s)\, h(s)\, ds = \dot{q}$, which can be handled according to the discussion of indicator dilution curves and which reduces to the classical Fick equation for $C_A(t)$ constant.

If flow is not constant, the system probably becomes nonlinear and the difficulties are those encountered in the previous discussion of nonlinear systems.

3. *Simultaneous uptake and release, and other complications.* When tissue uptake and tissue release occur simultaneously, combination of Equations 17 and 24 yields, for constant flow,

$$C_V(t) = \int_{-\infty}^{t} p(x)\, C_A(x)\, g(x, t - x)\, dx + \frac{1}{F}\int_{-\infty}^{t} \dot{q}(x)\, n(x, t - x)\, dx$$

Clearly, in this case the A-V difference gives little information about metabolic events at time t. Even in the steady state this is a difficult problem requiring more experimental data than are needed if only uptake or only release occurs. The problem falls into a somewhat different category than those distributive problems considered in the general development of equations here. In general, if the system is homogeneous (that is, if the concentration of the metabolite under study is the same in all venous blood draining the system), then in the steady state, $F(C_A - C_V)$ is the difference between uptake and release, uptake is measured by proper use of tracer quantities of labeled metabolite, and release is obtained by difference. If the system is not homogeneous, as in the forearm of man in which concentration of certain metabolites may vary widely from vein to vein, then this simple use of A-V differences of naturally occurring and isotopic metabolite is inadequate. The solution, if one is possible, requires some knowledge of the anatomy of the system and in general involves simultaneous A-V differences from more than one vein, and may also require simultaneous measurement of distribution of blood flow to various parts of the system and the use of A-V differences of more than one molecular species. More detailed exposition of this problem is reserved for another communication.

We have, however, raised the problem of tissue heterogeneity. Clearly it is worthless, in a system with many venous effluents, all with differing concentrations, to let all calculation of metabolism depend on measurement of concentration in blood from only one vein unless it is known that nearly all the blood leaving the system is drained by the vein sampled. In many experiments it is not possible to guarantee, as a result of anatomical good fortune or surgical intervention, that all venous blood flows through the vein sampled. It is therefore necessary to sample from a sufficient number of veins to be sure that most of the venous flow has been represented by the sampling, and it is necessary to know the contribution to total outflow made by blood flow through each bed represented by each vein sampled.

For the case of multiple exits from the system and heterogeneity of tissue metabolism, the flow which must be determined is not that through the whole system but only that through the fraction of the system drained by the vein sampled. Therefore, in defining the steady state it is not enough to demonstrate that total flow is constant but it must be shown that flow

through that part of the system represented by $C_V(t)$ is constant.

SUMMARY AND RECOMMENDATIONS

Rate of tissue metabolism, uptake or release, can be measured by multiplying blood (or plasma, whichever is appropriate) flow by arteriovenous difference only if flow is constant, arterial concentration is constant, and rate of tissue metabolism is constant. A further restriction is that if venous concentration is not identical in all veins draining the tissue the venous concentration used must be weighted by the relative contribution to total outflow made by the bed drained by the vein sampled.

If flow varies with time the distribution of transit times through the system is apt to be unmeasurable and the system cannot be handled by equations that depend on the superposition principle; that is, the system is apt to become a nonlinear nonstationary system. During such changes in flow there does not appear to be any reliable way to interpret the data. It is clear only that an increase in flow will probably lead to an overestimate of tissue uptake and a decrease in flow to an underestimate. These considerations eliminate the possibility of using A-V differences to measure metabolism when flow varies. As soon as flow has remained constant for a time equal to the longest transit time through the system, it can be regarded as satisfactory for use in estimation of metabolism. This time can be determined for each system by indicator dilution techniques. Phasic changes in flow about a stable mean can be regarded as constant provided the period is brief compared with mean transit time through the system.

With these restrictions in mind we assume in what follows that flow is constant. If either uptake or output is a function of time, the distribution of transit times through the system is not stationary but the system is probably satisfactorily represented as linear; that is, the transformations with respect to time of various inputs through the system are superposable. Because tissue metabolism is an event occurring within the system, the transit times from entrance to sites of metabolism or from sites of metabolism to exit are the ones we wish to measure but apparently can't at present. We can, however, plausibly relate the desired transit times to measurable transit times through the system. To find the metabolic rate at time t, clearly we need to use arterial concentration at some time before t and venous concentration at some time after t. For linear changes in uptake or output we are able to make these temporal corrections in arterial and venous concentration by using the approximate mean transit time which can be estimated by indicator dilution methods. For a nonmetabolized substance, mean transit time is its volume of distribution, V, divided by the flow F (plasma or blood water, in whichever it is dissolved) through the system. For most metabolites the appropriate volume of distribution is probably the total extracellular volume of the system. In the case of glucose, for example, mean transit time through the forearm of man in the basal state is about 10 minutes, which is a considerable correction on some time scales. The magnitude of the time correction also determines the frequency with which blood samples should be taken. It is probably prudent to sample at intervals no greater than the mean transit time unless arterial and venous concentrations are relatively stable or otherwise well defined.

If arterial concentration is also a function of time, the data are manageable if the function is well defined. This means that it must be measured often enough to define it. If the change in arterial concentration is linear with time, the correction is made by moving back in time to that arterial concentration, found or properly interpolated, that occurred one mean transit time unit earlier. Treatment of combinations of variations follows the same principle and is given in the body of this paper. The uncertainties are such that the investigator is urged wherever possible to design his experiments so that arterial concentration is held constant or so that he changes arterial concentration stepwise from one steady state to another. The time required to reach the steady state is not the mean transit time but the longest transit time through the system. For the case of glucose through the forearm of man in the basal state, the longest transit time is about 30 minutes.

In the introduction an operational definition of *constant* was promised. If the temporal cor-

rections proposed here do not lead to a corrected A-V difference that differs from the simultaneous A-V difference at time t by more than the experimental error of measurement of an A-V difference, it is probably safe to regard the functions as constant.

The effect of the non-steady state on simultaneous A-V differences means that a solitary A-V difference is uninterpretable. The immediate history of the system must be known. A single pair of A-V differences determined simultaneously on two substances passing through the same bed is also not properly useful for comparing the relative metabolism of the two substances unless it is known that the distributions of their transit times are identical.

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CORRECTION

On page 2000 of the article entitled "Studies of the Blood-Cerebrospinal Fluid Barrier to Cortisol in the Dog" by Nicholas P. Christy and Robert A. Fishman (J. clin. Invest. 1961, **40**, 1997), lines 9 and 10 in the right-hand column should read: "Samples of plasma and CSF were taken at hourly intervals. Constant levels of cortisol in plasma and CSF were attained after 4 hours and maintained for the succeeding 5 hours."

DEMONSTRATION OF CIRCULATING ANTINUCLEAR GLOBULINS IN ULCERATIVE COLITIS *

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Although Andresen (1) first considered "allergy" as a factor in the pathogenesis of ulcerative colitis in 1925, the recent studies of Levine, Kirsner, Klotz and Elchlepp (2–4) have renewed interest for the role of hypersensitivty in this disorder. Various clinical features suggest the presence of an altered immune state in ulcerative colitis. Among these are sensitivity to drugs and blood transfusion (5), the beneficial effects of ACTH and corticosteroids (6) and such extracolonic manifestations as erythema nodosum (7), uveitis (8), hepatitis (9, 10), myocarditis (6), glomerulitis (5, 11), arthritis (12–14), purpura (5), hemolytic anemia with positive Coombs test (15), leukopenia (16) and splenomegaly (8). In addition, a few reports have called attention to the presence of ulcerative colitis in patients with lupus erythematosus (17), scleroderma (18), or periarteritis nodosa (19).

Previous studies have shown the presence of circulating antinuclear globulins in patients with systemic lupus erythematosus as well as in cases of rheumatoid arthritis, complicated by splenomegaly and leukopenia (Felty's syndrome) (20). In the course of testing sera from patients presenting this clinical picture, two individuals with ulcerative colitis were found to have circulating antinuclear factors. The present study was undertaken to determine the nature and frequency of antinuclear globulins in ulcerative colitis. A preliminary report has been published (21).

MATERIALS AND METHODS

Human sera. Sera were obtained from 24 patients with proven ulcerative colitis and from 13 individuals who had previously undergone colectomy for this disorder. Four patients were studied before and after colectomy and are included in both groups. Over 300 sera from normal subjects and from patients with various disease states were tested while the study was in progress. Whenever multiple tests were performed, aliquots of the same serum sample were used. All sera were stored at −20° C.

Fluorescent antiglobulin tests. The details of the technique used have been reported previously (20). Briefly, the test sera were superimposed for 1 hour on: *1*) normal human peripheral blood smears fixed in 95 per cent ethanol (20), and on *2*) nucleohistone spots prepared from calf thymocytes (22, 23). The slides were thoroughly washed and fluorescein-labeled rabbit antihuman γ-globulin, absorbed with rat liver powder, was then used as a histochemical stain for human γ-globulin. The first procedure tests the affinity of the sera for human leukocyte whole nuclei and the fluorescent label is detected by ultraviolet microscopy. In the second method, the sera are tested against calf nucleoprotein and fluorescence is visible to the naked eye when the slides are exposed to the beam of a Woods lamp.

Lupus erythematosus (LE) cell preparations. The technique used was a variation of the Zinkham-Conley method (24). One ml of heparinized normal blood was added to 0.5 ml of test serum. After standing at room temperature for 2 hours with occasional shaking, 1.5 ml of the mixture was transferred to screw-top tubes containing glass beads and rotated at 40 rpm for 30 minutes at 37° C. The contents were then centrifuged in small-bore tubes to isolate the buffy coat which was smeared on glass coverslips, stained with Wright-Giesma and examined microscopically for the presence of LE cells.

Rheumatoid factor tests. Commercially available human γ-globulin-coated latex particles (Hyland RA test) were mixed with a 1:20 dilution of the patient's serum on a glass slide. Visible flocculation constituted a positive result.

Thyroglobulin antibodies. Commercially available thyroglobulin-coated latex particles (Hyland TA test) were mixed on glass slides with test sera. The sera were previously incubated at 56° C for 30 minutes and tested undiluted and at a 1:20 dilution. Macroscopic clumping constituted a positive result.

Serum protein determinations. Filter paper electrophoresis was performed in veronal buffer at pH 8.6 in a model R Spinco apparatus. The strips were stained with bromphenol blue and scanned with the Analytrol photo-

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TABLE I

Clinical and laboratory findings in patients with ulcerative colitis

Patients		Age, Sex	Duration of disease	ACTH-steroid therapy	Serum proteins A/G	Thyroglobulin antibody	Rheumatoid factor	LE prep.	Fluorescent antiglobulin test: Nucleoprotein	Fluorescent antiglobulin test: Whole nuclei	Remarks
1.	C.F.	27 ♀	8 yrs	Yes	2.6/2.8	0	0	0	0	+	Arthritis, splenomegaly, leukopenia
2.	J.L.	38 ♂	7 yrs	0	2.3/3.1	0	0	0	0	+	Arthritis, splenomegaly, leukopenia
3.	L.D.	40 ♂	23 yrs	0	2.48/2.64	0	0	0	0	+	Eosinophils 6%
4.	M.J.	45 ♀	4 mos	Yes	2.2/3.2	0	0	0	0	0	Alopecia
5.	M.S.	26 ♀	4 yrs	Yes	2.9/3.6	0	0	0	0	+	Patient and mother had rheumatic fever
6.	E.M.	55 ♀	2 mos	Yes	1.74/3.31	0	0	0	0	+	Disease developed after penicillin treatment
7.	B.A.	23 ♂	4 yrs	Yes	2.74/3.87	0	0	0	0	0	
8.	F.P.	17 ♂	1 yr	0	2.08/3.78	+	0	0	0	+*†	Hepatitis, arthritis. Aunt and cousin, ulcerative colitis
9.	J.G.	46 ♂	10 yrs	0	3.80/2.89	0	0	0	0	+	Psoriasis
10.	R.F.	28 ♂	8 yrs	Yes	1.55/4.14	0	0	0	0	+	Arthritis, splenomegaly, pleurisy, eosinophils 5%
11.	R.G.	19 ♂	1 yr	Yes	2.8/3.7	0	0	0	0	0	Arthritis, hepatitis, recurrent thrombophlebitis

* Test negative after ACTH-steroid therapy.
† Test negative after colectomy.

TABLE I—(*Continued*)

Patients		Age, Sex	Duration of disease	ACTH-steroid therapy	Serum Proteins A/G	Thyroglobulin antibody	Rheumatoid factor	LE prep.	Fluorescent antiglobulin test: Nucleoprotein	Fluorescent antiglobulin test: Whole nuclei	Remarks
12.	F.M.	35♂	2.5 yrs	0		0	0	0	0	+	Past history of arthralgias and hives
13.	R.M.	23♀	1 yr	Yes	2.3/4.2	0	0	0	0	+†	Allergic to penicillin
14.	J.H.	27♀	2.5 yrs	Yes	3.1/2.86	0	0	0	0	0	Arthritis, erythema multiforme nodosum
15.	M.H.	65♀	10 days	0	2.2/3.6	0	0	0	0	0	Leukopenia, eosinophils 6%
16.	J.P.	16♂	1 yr	Yes	2.73/5.76	0	0	0	0	+*	Dermatitis, penicillin allergy, splenomegaly, thromboses, pyroderma gangrenosum, glomerulitis, eosinophils 15%
17.	M.W.	45♂	15 yrs	0	3.72/3.66	0	0	0	0	+	Arthralgias, regional ileitis. Son, rheumatic fever
18.	A.L.	45♂	2 yrs	0	1.55/3.22	0	0	0	0	0†	Butterfly rash
19.	J.G.	42♂	25 yrs	0	3.80/3.32	0	0	0	0	+	Brother, rheumatic heart disease. Sister, nephritis
20.	C.M.	19♂	9 yrs	Yes	2.30/3.32	0	0	0	0	+	Arthritis. Mother, rheumatic heart disease
21.	M.W.	37♀	1 yr	0		0	0	0	0	+	
22.	S.R.	61♂	14 days	0	3.72/4.68	+	0	0	0	+	
23.	C.S.	57♂	1 yr	Yes		0	0	0	0	+	
24.	S.G.	18♂	2 yrs	Yes	2.82/3.73	0	0	0	0	+†	

densitometer. Normal values for this laboratory were: albumin, 3.39 to 5.59 g; globulin, 1.88 to 4.08 g.

RESULTS

The pertinent clinical and laboratory findings in 24 patients with ulcerative colitis are listed in Table I. Positive fluorescent antiglobulin tests against whole nuclei were observed in 18 of these patients (Figure 1A). However, uniformly negative results were obtained when sera were tested for affinity to calf thymus nucleoprotein (Figure 2), for the presence of the rheumatoid factor or for their ability to induce LE cells. Thyroglobulin antibody tests were negative in all but two individuals, both without evidence of thyroid disease. Serum protein determinations indicated borderline elevation of serum globulins in four subjects and frequent hypoalbuminemia, a common finding in ulcerative colitis. Positive antinuclear tests were not related to age, sex or history of hypersensitivity disorders, although frequent manifestations of the latter were present in several of the patients or their relatives. Approximately half of the patients were receiving ACTH or corticosteroid therapy at the time of testing, and in one-third the disease had been present for over 5 years.

The results of the fluorescent antinuclear test with respect to certain clinical findings are reported in Table II. The over-all results of the test in this series of patients did not seem to be greatly influenced by ACTH or corticosteroid therapy. Nine of 13 individuals under treatment had positive tests as compared with 9 of the 11 patients not receiving hormones. However, in two individuals (Table I, Subjects 8 and 16), the fluorescent antinuclear tests became negative during hormone therapy. In 9 of 13 postcolectomy patients, including two individuals who had positive tests before operation and another who had reverted to negative on hormone therapy, no antinuclear globulins were detected (Table I). In the group of patients whose disease manifestations

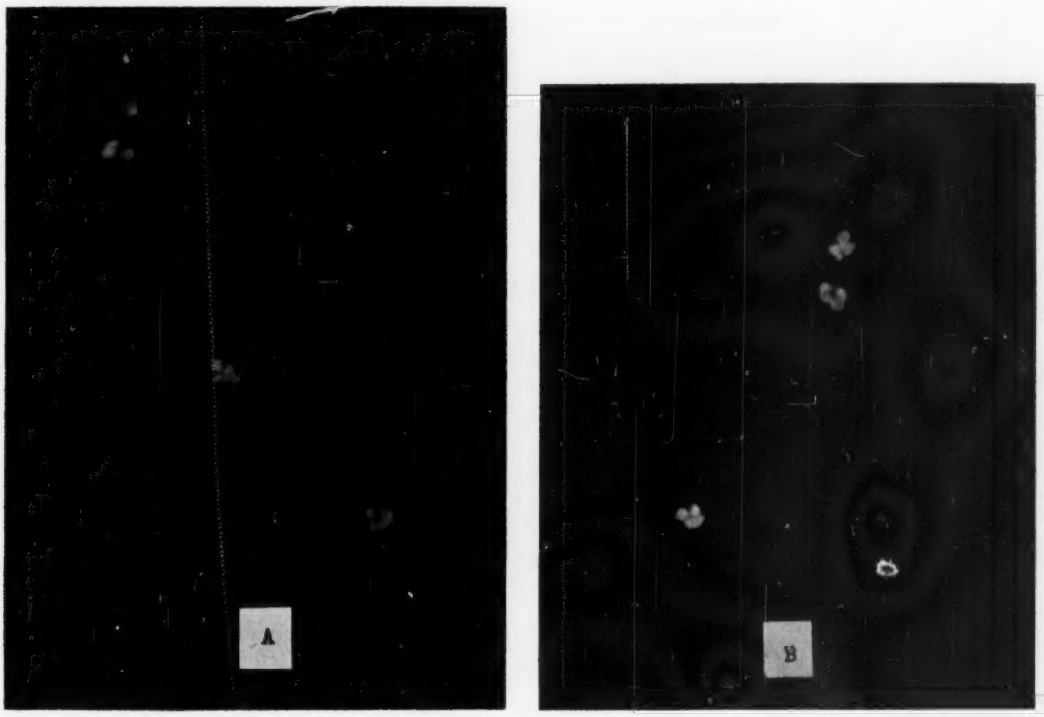

FIG. 1. A) POSITIVE FLUORESCENT ANTIGLOBULIN TEST AGAINST HUMAN LEUKOCYTE WHOLE NUCLEI PREVIOUSLY EXPOSED TO SERUM OF A PATIENT WITH ULCERATIVE COLITIS. B) SIMILAR REACTION WITH SERUM FROM A PATIENT WITH LUPUS ERYTHEMATOSUS. Note brighter fluorescence.

FIG. 2. FLUORESCENT ANTIGLOBULIN TEST AGAINST CALF THYMUS NUCLEOPROTEIN SPOTS PREVIOUSLY EXPOSED TO TEST SERA. Negative reaction (left) with ulcerative colitis serum; positive reaction (right) with lupus erythematosus serum.

had been present for less than 5 years, ten had positive and six had negative antinuclear tests as compared with the eight individuals with a history of over 5 years' duration, all of whom were found to have circulating antinuclear globulins. All four patients with splenomegaly and seven of the nine who suffered joint manifestations had positive antinuclear tests.

Table III lists the results obtained to date with sera tested for antinuclear factors from a group of normal controls and from patients with a variety of disease states. Certain specific disorders, included in this list for comparison, have formed the basis for or have been included in part in earlier reports and are designated by appropriate references (20, 25–27). In every case, the sera studied for affinity to calf thymus nucleoprotein were also tested against fixed whole nuclei of normal peripheral blood leukocytes, but in some cases only the latter test was performed. Circulating antinuclear factors have been detected by these methods in lupus erythematosus, rheumatoid arthritis and related disorders, as well as in chronic liver disease and in some drug reactions.

TABLE II

Correlation of antinuclear test with clinical findings in ulcerative colitis

Clinical finding	Fluorescent antiglobulin test (whole nuclei)	
	No. positive	No. negative
Arthritis and arthralgia	7	2
Splenomegaly	4	0
Duration of disease		
Under 5 yrs	10	6
Over 5 yrs	8	0
Postcolectomy	4	9
ACTH-steroid therapy		
Yes	9	4
No	9	2

DISCUSSION

In addition to the clinical observations previously mentioned, some support for a possible relationship of ulcerative colitis to the hypersensitivity states stems from a few recent experimental studies. Broberger and Perlmann (28) demonstrated that sera from patients with ulcerative colitis contained precipitating and hemagglutinating factors capable of reacting with tissue extracts from colon as well as from liver or kidney. Furthermore, the same authors observed absorption of γ-globulins from these sera onto colonic cells in tissue culture by fluorescent techniques, but did not describe nuclear or cytoplasmic localization, specificity, or prevalence of this reaction. Polcak and Vokurka (29) studied a circulating factor which agglutinated collodion particles coated with extracts of normal colonic mucosa and submucosa. Bregman and Kirsner (30) report detecting circulating antibodies apparently specific for colon mucosa by double agar diffusion and hemagglutination techniques.

The results of the present investigation demonstrate that a circulating antinuclear γ-globulin is present in many patients with ulcerative colitis. It is important to emphasize that the fluorescent antiglobulin technique, as used in this study, serves only as a histochemical method for detecting globulins and does not distinguish antibodies from abnormal proteins which may have affinity for nuclear material. The inability to determine a specific antigen, as well as the absence of additional information regarding the nature of these antinuclear globulins and their behavior in other immunological systems, precludes, for now, the conclusion that they are true antibodies or that immunological mechanisms are involved in their reactions with nuclear material. Furthermore, the relation of this factor to those detected by other workers remains to be established. In our system this factor was detected on homologous leukocyte nuclei in a reaction quantitatively similar to that observed with sera of some patients with

TABLE III

Results of tests for antinuclear factors in sera of individuals without ulcerative colitis

Diagnosis	Fluorescent antibody test			
	Nucleoprotein		Whole nuclei	
	No. tested	No. positive	No. tested	No. positive
Systemic lupus erythematosus (20)	12	12	21	21
Rheumatoid arthritis (25)	50	5	50	17
Other collagen diseases	5	2	5	5
Hepatitis and cirrhosis (26)	19	3	65	24
Leukemias and lymphomas (27)	30	0	30	0
Drug reactions	7	0	7	2
Carcinoma of colon	17	0	17	0
Other diseases of colon*	9	0	9	1†
Other diseases (20)	62	0	108	0
Normal laboratory personnel	15	0	15	0
Totals	226	22	327	70

* Tests negative in: amebic colitis, 2; mucous colitis, 2; diverticulitis, 2; schistosomiasis, 1; multiple polyps, 1.
† Positive antinuclear test in man with familial polyposis whose son has ulcerative colitis.

rheumatoid arthritis (25), chronic hepatitis (26), drug reaction, scleroderma, polyarteritis, and in the sera of patients with lupus erythematosus in relative remission. Higher titers of this factor are usually encountered in patients with rheumatoid arthritis accompanied by complications such as splenomegaly and leukopenia, and in individuals with active lupus erythematosus (20, 25). In over 250 sera from healthy subjects as well as from patients with other diseases characterized by leukopenia, hyperglobulinemia, splenomegaly or colon pathology, antinuclear globulins were not demonstrable.

While many of our patients exhibited some of the features often attributed to hypersensitivity disorders, none fulfilled the clinical picture of systemic lupus erythematosus. The LE cell preparations, in agreement with the findings of Lagercrantz, Winberg and Zetterström (5), were uniformly negative. The antinucleoprotein test, which has shown closer correlation with the LE phenomenon than the reaction against whole nuclei (25), was also negative in every case. In accord with other reports (12, 13), the rheumatoid factor was absent in all of our patients with ulcerative colitis even when arthritis was present. Thyroglobulin antibodies were present in two of our patients, but this finding has been reported in 18 per cent of a normal hospital population (31).

Although the number of cases is too small to warrant definite conclusions, attention should be called to the fact that patients with splenomegaly, arthritis or arthralgia, or disease of over 5 years' duration usually had positive antinuclear tests (Table II). While, in general, postcolectomy cases gave negative results, this serum factor was present in nine patients on ACTH-corticosteroid therapy as well as in nine patients not receiving hormones. At the present time we do not know what may be spontaneous fluctuations of these factors during the natural course of the disease, the influence of hormones, or the role played by the presence of a diseased colon.

The possible implications of the presence of circulating antinuclear globulins in ulcerative colitis should be tempered by the realization that little is known about their significance or about the nature of the diseases in which they are present. No one has yet demonstrated that these abnormal globulins play a specific role in the pathogenesis of any of the disorders in this group (32). A recent report (33) that clinically healthy relatives of patients with lupus erythematosus have circulating antinuclear globulins suggests the possibility that this is a genetically controlled protein abnormality which merely denotes or accompanies an unusual type of immunological reactivity. In some individuals with this constitution, exposure to unknown stimuli might result in different clinical syndromes with some common features.

Whether this circulating antinuclear globulin in the individual with ulcerative colitis represents an increased susceptiblity for, a primary factor in, or

an incidental result of the disease process remains to be determined.

SUMMARY

Sera from 24 patients with ulcerative colitis were studied for the presence of antinuclear globulins by the fluorescent antiglobulin technique. In 18 patients (75 per cent) a positive reaction was detected on whole nuclei of human leukocytes, but no affinity for calf thymus nucleoprotein was observed. Tests for rheumatoid factor and lupus erythematosus cell induction were negative.

Antinuclear factors were usually present in patients with splenomegaly, arthritis or arthralgia and long-standing disease (over 5 years). They were absent in 9 of 13 patients who had previously undergone colectomy for their disorder, including 2 who had positive tests before surgery. While positive antinuclear tests were present in 9 patients on ACTH-corticosteroid therapy, in 2 the test became negative during treatment.

Over 300 sera from normal subjects and patients with various diseases were tested. Positive antinuclear tests were encountered in systemic lupus erythematosus, rheumatoid arthritis and related collagen disorders, as well as in chronic hepatitis, cirrhosis and in some drug reactions.

The presence of a circulating antinuclear globulin in ulcerative colitis is of interest, since many patients with this disease exhibit features often attributed to hypersensitivity disorders.

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DETERMINATION OF HUMAN PLATELET SURVIVAL UTILIZING C^{14}–LABELED SEROTONIN *

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All of the methods available for estimation of platelet survival have certain disadvantages. Isolation and considerable manipulation of platelets *in vitro* are necessary with the Cr^{51} technique (1, 2). The P^{32} (3), diisopropylfluorophosphate^{32} (DFP^{32}) (4), or S^{35} (5) methods are all *in vivo* labeling procedures. Studies with P^{32} or S^{35} require special subjects for study (patients with polycythemia vera or leukemia). Finally, both autologous and homologous platelet survival cannot be studied with facility.

The use of serotonin [5-hydroxytryptamine (5-HTA)] as a platelet label appeared to have certain advantages. It is preferentially concentrated by blood platelets *in vivo* and *in vitro* (6). Isolation of platelets is not required to achieve an *in vitro* label, and *in vivo* labeling of platelets can be accomplished without labeling their precursors. We have used radioactive labeled (C^{14}) serotonin to study platelet survival.

MATERIALS AND METHODS

C^{14}-labeled 5-hydroxytryptamine.[1] C^{14}-labeled 5-HTA [5-hydroxy-3-indolyl-(ethyl-2-amino-1-C^{14})] creatinine sulfate monohydrate complex (specific activity 6.87 mc per mmole) was made up in sterile, pyrogen-free, isotonic saline solution in concentration of 2.5 μc per ml. Each milliliter of solution contained approximately 64 μg of 5-HTA. After filtration through a cellulose bacteriological [2] filter and bacterial culture, the solution was stored in a refrigerator at 4° C in a multi-dose vial. Paper chromatography of the material as originally received confirmed that greater than 95 per cent of C^{14} activity was associated with 5-HTA. Deterioration of the substance was noted on prolonged storage. After 3 months only 50 per cent of the C^{14} activity was still associated with 5-HTA.

We were initially concerned with the pharmacological and radiation hazards of C^{14}-5-HTA. On the basis of *in vitro* studies, it was determined that a usable label for *in vivo* studies could be achieved with 1.0 to 2.0 μc of C^{14}-5-HTA. 5-HTA has profound physiological effects when given intravenously in amounts of 2 mg or more (7). In these studies, however, the amount given is much smaller (25 to 50 μg) and in greatest part is bound to platelets and unavailable for immediate pharmacological action. Local venospasm has not been observed in any of the experimental subjects. The radiation hazard is negligible. The maximal permissible body burden of C^{14} is 400 μc (8). In these studies no more than 2 μc was used. Since it is probable that the end-products of 5-HTA catabolism are excreted practically *in toto*, it seems unlikely that C^{14} injected as 5-HTA would enter other metabolic pathways to any appreciable extent (9).

Preparation of C^{14}-5-HTA-labeled platelets. Blood, 250 ml, was drawn directly (via a 16 gauge needle) into a plastic bag. Initially, 30 ml of 0.1 M sodium citrate was prepared and used as the anticoagulant, because Born and Gillson had indicated (10) that uptake of 5-HTA by platelets was maximal under these circumstances. Subsequently, commercially prepared plastic bags (Cutter) containing acid citrate dextrose as the anticoagulant have been used with results similar to those obtained with 0.1 M sodium citrate. After withdrawal of the blood, 1 to 2 μc of C^{14}-5-HTA solution was injected into the bag and the mixture was incubated at 37° C for 30 to 45 minutes in a water bath with occasional gentle swirling. At the end of the incubation period the contents of the bag were rapidly infused into the experimental subject, retaining approximately 15 ml in siliconized conical volumetric centrifuge tubes for sampling of platelets and plasma for radioactivity.

Determination of amount of C^{14} activity in the infused platelets. A hematocrit determination and platelet counts using the method of Brecher and Cronkite (11) were done on the retained sample. The remainder of the sample was then immediately centrifuged and platelet-rich plasma obtained. The platelet-rich plasma was separated and platelet counts performed. A 2-ml aliquot of platelet-rich plasma was transferred to a sample container (20-ml liquid scintillation counting bottle). The remaining volume of platelet-rich plasma was measured and centrifuged to obtain a platelet plug. The plate-

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[1] Nuclear-Chicago Corporation.

[2] Millipore type HA filter, Millipore Corporation.

let-poor plasma was decanted from the plug and 2 ml transferred to a sample container. The silicon-coated conical tubes containing the residual platelet plug were inverted and the excess plasma allowed to drain thoroughly. One ml of distilled H_2O was added to the platelet plug and the plug dispersed using an ultrasonic vibrator.[3] This last step yielded a homogeneous suspension which was decanted into a third sample bottle. One ml of distilled H_2O was used as a washout of the tube and added to the sample container to bring the total volume of the disintegrated platelet suspension to 2 ml. Approximately 0.6 to 1.0×10^9 platelets were suspended in 2 ml of platelet-rich plasma and 1.0 to 1.5×10^9 platelets made up the platelet plug obtained from normal subjects. The platelet-poor plasma rarely had more than 0.003×10^9 platelets in the 2-ml aliquot (corresponding to fewer than 1,500 platelets per mm^3 of plasma).

The sample bottles into which the 2-ml volumes of platelet-rich plasma and the dispersed platelet plug were decanted had been previously prepared by the addition of 18 ml of a liquid scintillation mixture of dioxane, anisole and dimethyloxyethane (6:1:1 by volume) containing 7 g of PPO (2,5-diphenyloxazole) and 50 mg of POPOP [2,2-*p*-phenylenebis (5-phenyloxazole)]. In order to maintain the intact platelets in suspension, this mixture had been modified by addition of Thixcin[4] in concentrations of 2.5 g per 100 ml to form a gel. Counting efficiency in a liquid scintillation counter[5] with this system was approximately 30 per cent for aqueous colorless solutions. Efficiency was lower (approximately 20 per cent) for the samples containing colored solution (i.e., plasma). Samples were counted long enough to achieve a counting error of 3 per cent or less (usually 10 to 30 minutes). The use of liquid scintillation counting methods for *in vivo* studies was dictated by the relatively low C^{14} activity in platelets after dilution in the blood volume of the study subjects had occurred. The major advantage was in the number of platelets which could be conveniently placed in a liquid counting system as opposed to solid sample beta counting systems. Recovery of radioactivity could be enhanced by extraction of C^{14}-labeled serotonin from platelets or by use of a more efficient liquid counting system. The method used, however, allows rapid preparation of samples and is adequate in terms of efficiency.

C^{14}-5-HTA in red cells was not counted directly. *In vitro* experiments confirmed previous findings that red blood cells concentrate and retain 5-HTA poorly (6). It is unlikely that the small number of red cells in the samples contributes any of the radioactivity observed. Further confirmation of this point came from the use of reference standards for the total amount of radioactivity infused. Virtually all of the injected activity could be accounted for by totaling platelet and plasma activity.

Sampling of the recipient. Fifteen ml of whole blood was drawn with silicon-coated equipment and processed in the same manner as the aliquot of infused bag blood. The recipient was sampled until the radioactivity per 10^9 platelets was less than 20 per cent of the amount present 24 hours after infusion. In addition, 24-hour urines were collected from some subjects and total C^{14} activity in the urine determined.

Experimental subjects. Eleven normal males who had never received blood transfusions were infused with C^{14}-labeled platelets. Two experiments in normals were cross-transfusion studies. The remaining experiments were infusions of the subject's own labeled platelets. An additional normal subject received C^{14}-labeled 5-HTA infused in saline rather than in whole blood to determine the efficiency of *in vivo* labeling. Finally, four patients with thrombocytopenia were studied on five occasions. Three of these patients received homologous platelet infusions. The fourth patient was reinfused with his own platelets that were labeled *in vitro*, as in the normal subjects.

RESULTS

Yield of infused platelets. In nine experiments in normal subjects, it was possible to calculate the expected level of radioactivity in the circulating platelets following infusion of C^{14}-5-HTA-labeled platelets. Two experiments lacked data essential for this calculation. The radioactivity in circulating platelets was determined for each sampling period and the per cent yield was calculated according to the formula:

$$\text{per cent yield} = \frac{(\text{cpm, platelets observed})\ (\text{platelets/ml})\ (\text{blood vol, ml})}{(\text{cpm, platelets infused})\ (\text{platelets/ml})\ (\text{ml infused})} \times 100$$

Per cent yield for 2, 6 and 24 hours, summarized in Table I, included seven autologous and two homologous platelet infusions. The lowest yield, 39 per cent (Subject SH), differs from the re-

TABLE I

Per cent of calculated expected yield observed at 2, 6, and 24 hours in nine normal subjects

Subject	Time		
	2 hrs	6 hrs	24 hrs
	%	%	%
SH	39	49	36
KY	80		62
HB	75	80	75
LF	73		65
BD	54	58	51
NF	47		47
PF	51		59
EG*	59		61
JP*	56		57
Mean	59	67	57

* Homologous platelet infusions.

[3] Narda "Sonblaster."

[4] Baker Castor Oil Company.

[5] Packard Tri-Carb.

TABLE II

Efficiency of in vivo labeling compared with in vitro labeling

Subject	Cpm/10^9 platelets at 24 hrs	Infused C^{14} activity in platelets at 24 hrs	C^{14} activity excreted at 24 hrs
		% *total*	%
RH (*In vivo* label)	61	7	53
EG (*In vitro* label)	385	61	8
JP (*In vitro* label)	630	57	6

mainder. This study was done with 75 ml of blood drawn into a silicon-coated glass bottle and incubated with C^{14}-5-HTA as usual. Unfortunately, it was necessary to reinject the blood with multiple syringes. The remainder of the experiments all utilized greater volumes of blood drawn into plastic bags and reinfused directly into the recipient. The lowest yield of the last eight experiments was 47 per cent. The mean yield for all nine experiments was 59 per cent at 2 hours and 57 per cent at 1 day. The results in two subjects (EG and JP) who received homologous platelets were similar to those of the autologous platelet infusions. The rise to peak activity at approximately 24 hours, seen in other studies (1–3) and interpreted as indicating temporary platelet sequestration *in vivo*, was not observed in these experiments. A small rise occurred in many between 2 and 6 hours. However, the 2-hour value usually was similar to or higher than the 24-hour value.

Calculation of per cent yield is subject to error since C^{14}-5-HTA free in plasma is present in the infusion, and *in vivo* labeling of platelets occurs. The magnitude of this error was assessed by infusing a normal subject with C^{14}-5-HTA in 250 ml of 0.85 per cent sodium chloride solution. Experiments done in Subjects RH, EG and JP are summarized in Table II. The blood of EG and JP was typed, cross-matched and cross-transfused after their platelets were labeled in plastic bags, as previously described. Less than 10 per cent of the infused C^{14} activity was free in plasma; the remainder was in platelets. RH received a similar amount of C^{14}-5-HTA in a volume of saline comparable to the whole blood infusions received by EG and JP. Less than 10 per cent of the infused

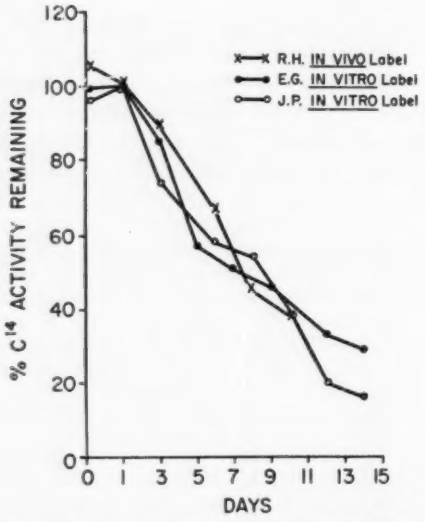

FIG. 1. DISAPPEARANCE OF C^{14}-LABELED 5-HTA FROM PLATELETS. EG and JP received *in vitro* labeled homologous platelets. RH was infused with 5-HTA in saline, an *in vivo* platelet label. 100 per cent = counts per minute per 10^8 platelets at Day 1.

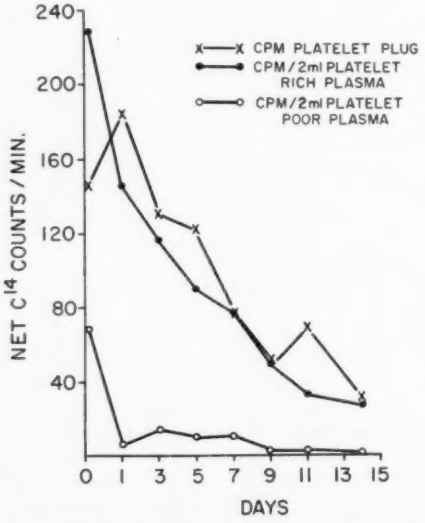

FIG. 2. COMPARISON OF C^{14} ACTIVITY IN PLATELET-RICH PLASMA, PLATELET-POOR PLASMA AND PLATELET PLUG. The total number of platelets in samples of platelet-rich plasma was approximately 0.7 to 0.8×10^9; in platelet-poor plasma 0.004×10^9; in the platelet plug 0.7 to 0.8×10^9 platelets.

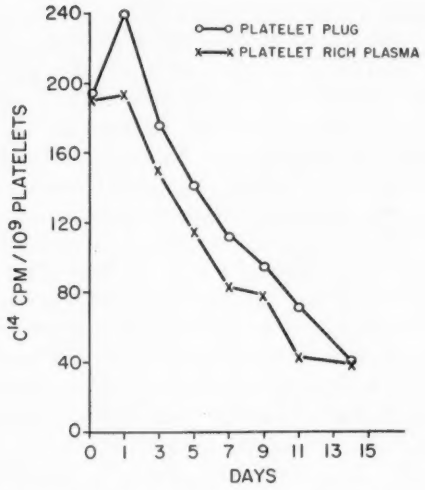

FIG. 3. PLATELET SPECIFIC ACTIVITY CALCULATED BY USING COUNTS PER MINUTE OBSERVED IN THE PLATELET PLUG AND IN PLATELET-RICH PLASMA. The lower specific activity seen in platelets suspended in platelet-rich plasma is due to the quenching effect of plasma as opposed to the aqueous platelet plug sample.

activity was present in urine (EG and JP) at the end of 1 day when C¹⁴-5-HTA bound to platelets was infused. Fifty-three per cent of infused activity was in the 24-hour urine of the subject (RH) receiving C¹⁴-5-HTA in saline. *In vivo* labeling of the platelets of RH was appreciable. This was, however, less than 20 per cent of the activity found when a comparable amount of 5-HTA labeled to platelets was infused. Since usually no more than 10 per cent of the injected activity is in plasma, free to label circulating platelets in the recipent, the magnitude of the error introduced by *in vivo* labeling is probably small. Overestimation of per cent yield by no more than 3 to 5 per cent results. The disappearance rates of the C¹⁴-5-HTA from the circulating platelets of the three subjects were similar (Figure 1).

Platelet survival in normal subjects. Counts per minute per 2 ml of platelet-rich plasma, platelet-poor plasma, and counts per minute in the platelet plug are plotted against days for a typical experimental subject (Figure 2). Comparatively, the amount of activity in platelet-poor plasma was small. However, in calculating platelet specific activity, the platelet-rich plasma counts are corrected by subtracting platelet-poor plasma counts, because all of the C¹⁴ activity present in plasma on the first day cannot be accounted for by activity present in the few platelets which have remained in suspension in platelet-poor plasma. C¹⁴ platelet specific activity calculated by use of values obtained from the corrected platelet-rich plasma and platelet plug is plotted against days in Figure 3. Platelet specific activity based on the platelet plug is higher. This can readily be accounted for by the greater quenching effect of plasma in the scintillation solution as opposed to the aqueous platelet plug solution. In this case the quenching effect of the plasma resulted in reduction of observed counts to approximately 80 per cent of total counts in a colorless aqueous solution, as estimated by using internal standards in the scintillation solution. Correction of observed counts per minute per 10^9 platelets derived from platelet-rich plasma results in close agreement of the two values for platelet specific activity.

It is possible to express per cent survival as a

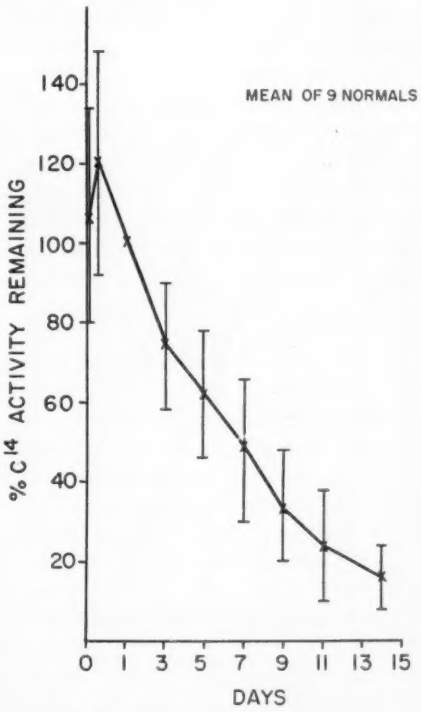

FIG. 4. MEAN ± 2 SD OF NINE AUTOLOGOUS C¹⁴-LABELED PLATELET INFUSIONS. 100 per cent activity is taken arbitrarily as platelet specific activity at 24 hours.

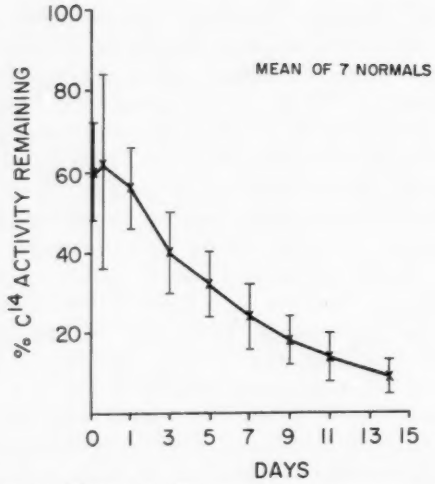

FIG. 5. MEAN ± 2 SD OF SEVEN AUTOLOGOUS C^{14}-LABELED PLATELET INFUSIONS. 100 per cent = calculated expected yield.

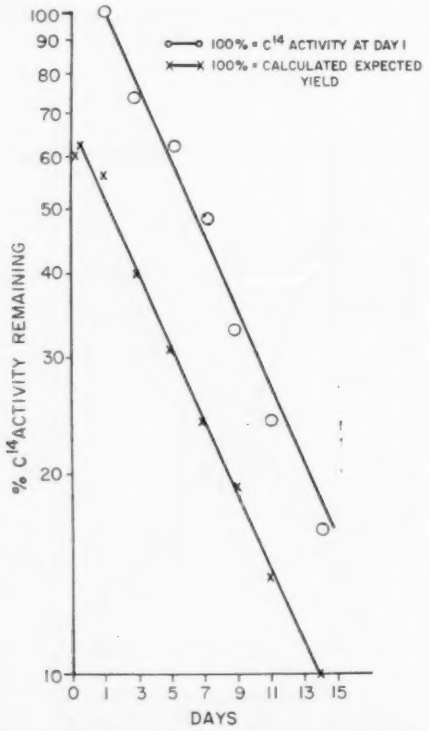

FIG. 6. VALUES FROM FIGURES 4 AND 5 PLOTTED ON SEMILOGARITHMIC COORDINATES. 100 per cent = value at Day 1; mean of 9 experiments (○——○). 100 per cent = calculated expected yield; mean of 7 experiments (×——×).

function of time in two ways: either as a per cent of activity per 10^9 platelets at some arbitrary time after infusion, or as a per cent of the calculated expected yield, as Aas and Gardner (1) and others (2) have done. The latter method has the advantage of compensating for changing platelet counts in the recipient. Figure 4 illustrates the mean, plus or minus 2 standard deviations, of nine experiments done in normals with autologous transfusions. Specific activity 24 hours after infusion has been arbitrarily taken as 100 per cent. Figure 5 represents seven of these nine experiments with per cent activity remaining plotted against time as a function of platelet yield. Figure 6 shows the data of Figures 4 and 5 plotted on semilogarithmic coordinates. An exponential disappearance of C^{14}-serotonin from the circulating

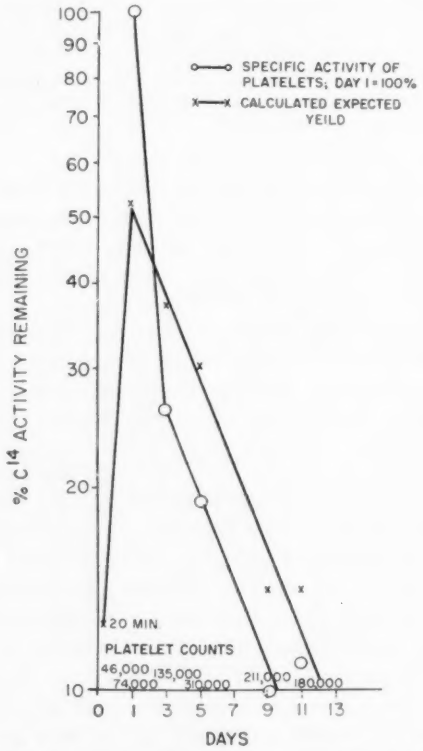

FIG. 7. PLATELET SURVIVAL IN DRUG-INDUCED (?) THROMBOCYTOPENIA IN RECOVERY PHASE. Survival (○——○) based on platelet specific activity appears markedly shortened initially, while the curve (×——×) based on calculated expected yield is basically normal. Platelet counts in peripheral blood at time of sampling are indicated.

TABLE III

Per cent of circulating platelet radioactivity, expected and observed platelet rise, and urinary excretion of C^{14} in four homologous platelet transfusions in thrombocytopenia

Subject	Yield at 24 hrs	Expected platelet yield/mm³	Observed platelet yield/mm³	Per cent of expected platelet rise observed	C^{14} excreted first 24 hrs
	%			%	%
EJ (DLE)	53	22,000	11,000	50	47
EC (Acute leukemia)	5	65,000	0	0	49
MD (ITP)					
1	4	7,650	0	0	80
2*	6	148,000	(?)2,000		92

* Infused with blood from patient with idiopathic thrombocythemia (platelet count of donor 1.5×10^6/mm³).

platelet mass was seen. The half-times for these curves were close to 5 days in both cases. They are similar to the *in vivo* and homologous labeled curves (Figure 1). One difference is in the time necessary for the curves to reach 20 per cent or less of initial activity. In the case of the per cent yield model, this occurred at approximately 8 days, while less than 20 per cent was not reached until 13 or 14 days after infusion, if all values are referred to the amount of activity present 24 hours after infusion.

The advantage of using per cent of calculated platelet yield is best illustrated (Figure 7) by a patient who developed acute thrombocytopenia, possibly drug-related (Librium [6]). A platelet survival study, utilizing the patient's own platelets, was initiated 2 days after steroid therapy was begun. Peripheral blood platelet count at that time was 46,000 per mm³. The curve based on per cent yield indicates a basically normal survival ($T_{\frac{1}{2}} = 5$ days), while that based on per cent residual activity per 10^9 platelets is initially suggestive of a markedly shortened survival. The impression of a relatively normal life span is further strengthened by the slope of the curve based on platelet specific activity after 3 days, when marked changes in platelet count were no longer occurring. This portion of the curve has a half-value of 5 days much like that in the normal situation.

[6] Roche; 7-chloro-2-methylamino-5-phenyl-3H-1,4-benzodiazepine 4-oxide hydrochloride.

Platelet survival in thrombocytopenia. One of the studies in patients with thrombocytopenia has been referred to in the preceding section and was done with autologous platelets. The remaining three patients were studied a total of four times with homologous infusion of platelets. These included a 34 year old female with disseminated lupus erythematosus (DLE) and thrombocytopenia, a 54 year old male patient with acute leukemia in relapse and severe hemorrhagic manifestations who had previously received multiple transfusions, and two studies done in a 31 year old female patient with acute idiopathic thrombocytopenic purpura (ITP). Per cent yield at 24 hours, expected platelet rise, observed platelet rise, and per cent platelet rise, as well as C^{14} urinary excretion data are summarized for these three patients (Table III). The patient with idiopathic thrombocytopenic purpura had developed purpura 2 weeks prior to study with peripheral blood platelet counts of around 3,000 per mm³. She had received no blood transfusions at the time of these studies. The massive destruction of platelets previously observed (12) in idiopathic thrombocytopenic purpura was corroborated. The patient with leukemia had massive purpura at the time of study and had received multiple transfusions before the study was done. Rapid utilization of platelets due to his hemorrhagic diathesis, or destruction of platelets due to antibodies induced by prior transfusion of platelets, or both, could account for the result seen. The patient with lupus erythematosus had a defi-

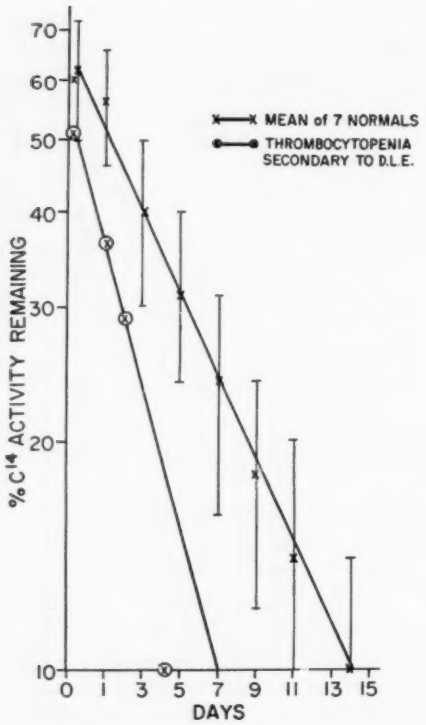

FIG. 8. PLATELET SURVIVAL IN A PATIENT WITH THROMBOCYTOPENIA (PLATELET COUNT 33,000 PER MM^3) SECONDARY TO DLE.

nitely shortened platelet survival with a half-life of less than 3 days. Platelet survival in this patient is plotted and contrasted with the seven autologous infusions done (Figure 8). The result may indicate simply more rapid utilization of platelets because of a decreased circulating platelet mass. Another possibility is a circulating platelet antibody due either to the basic disease or to three transfusions of whole blood received 4 months previously. The patient did not have a positive direct or indirect Coombs' test at the time of this study.

DISCUSSION

These studies were initiated because Udenfriend, Weissbach and Bogdanski noted the close correlation between the half-time of 5-HTA in platelets and platelet survival in rabbits and dogs (13, 14). The studies cited here in humans and similar studies by us in rats (15) extend these observations to two other species.

Still lacking is direct experimental proof that 5-HTA, once taken up and perhaps bound in platelets, is not lost *in vivo* until platelet lysis occurs. Born and Gillson (10) have shown that exchange of C^{14}-labeled for nonlabeled 5-HTA can occur between plasma and platelets *in vitro*. However, the conditions of their experiment would rarely, if ever, be found *in vivo*, as they properly pointed out. A study is cited by Zucker (16) on platelet 5-HTA disappearance rate in a patient with the carcinoid syndrome. Platelet 5-HTA disappeared in approximately 2 hours. The difference between that situation and our results is unexplained. It may relate to the basic disease process in the carcinoid syndrome.

The possibility exists that agreement between the half-time of disappearance of 5-HTA from circulating platelets and estimates of platelet survival *in vivo* is coincidental. We have been unable to devise a direct method for settling this point, other than radioautography of platelets. In humans, using the present amounts of C^{14}-labeled 5-HTA, it would take an estimated 40 years' exposure of the radioautographic material before a definite platelet label was detectable. We are now doing these studies in rats, since they have a much smaller platelet mass and a much heavier platelet label can be achieved. Another possibility is homologous infusion of labeled platelet-rich blood into an individual with thrombocytopenia due to marrow megakaryocyte aplasia and study of concomitant platelet and C^{14} disappearance rates.

Loss of platelet label from the circulation, studied by this method, is clearly exponential. Recently, much work has been done by several groups using various isotopic techniques to study the survival of platelets in the circulation in humans and animals. Disappearance of platelets from the circulation with the DFP^{32} (an *in vivo* label) method of Leeksma and Cohen (4) is reported as a linear phenomenon. Odell and Anderson (5), using S^{35} *in vivo* labeling of platelets in rats and humans, also have reported linear disappearance rates, but pointed out that the derived curves could as well be drawn as exponentials. Initially, Aas and Gardner (1) stated that the disappearance of platelets labeled *in vitro* with Cr^{51} was a linear phenomenon. In a more recent publication, however, Cohen, Gardner and Barnett

(17), using the Cr^{51} technique, have described the disappearance rate of platelets from the circulation as the sum of two expo.. .ntials multiplied by two constants rather than as a linear phenomenon. Adelson and co-workers (18) labeled platelets of polycythemic individuals *in vivo* with P^{32} and infused them into normal recipients. Exponential disappearance curves of platelet radioactivity were obtained. They have interpreted their results to indicate "utilization of platelets" in a constant process of *in vivo* clotting. They further presented evidence to show that platelet survival curves by their method can be altered from a clearly exponential function to a linear disappearance rate by anticoagulation with coumarin derivatives. Because of uncertainties with regard to re-utilization of C^{14}-5-HTA label, exchange of tracer between labeled and nonlabeled platelets or between labeled platelets and other body pools of serotonin, the question of whether platelets are utilized (random destruction) or age and die in the circulation cannot be settled from these studies.

The half-life of survival of platelets in the circulation has ranged, by these various methods, from 2 to 3 days (17, 18) to 5 days (2, 4, 5). Total life span has been estimated at from 8 to 14 days, the highest value being an upper limit reported by Pollycove, Dal Santo and Lawrence (19) using DFP^{32}. The results with C^{14}-5-HTA are in reasonable agreement with these estimates.

The yield of infused platelets of approximately 57 per cent observed at 24 hours is in accord with the concept of a relatively small reserve of noncirculating platelets (20). It has been estimated that the noncirculating platelet mass is only one or perhaps two times greater than the circulating platelet mass. Alternatively, the possibility exists that some of the infused platelets fail to circulate because of damage induced by manipulation. Platelet sequestration described by others is not a constant or a striking phenomenon in these studies. In normal subjects, the 24-hour period after injection appears to be more one of equilibration and redistribution than an active sequestration of platelets. Marked sequestration was noted in two abnormal subjects, one with drug-induced thrombocytopenia and one with lupus erythematosus. These differences again are unexplained.

The method described has several advantages over others. The most important is ease and simplicity of the labeling procedure and the minimal manipulation of platelets required. Either *in vitro* or *in vivo* labeling of platelets can be accomplished. Autologous or homologous studies may be done. The greatest disadvantage is the need for liquid scintillation counting techniques when studying as large an animal as man and the relatively expensive instrumentation required.

SUMMARY

1. The disappearance rate of C^{14}-5-HTA (5-hydroxytryptamine) from circulating platelets in normal subjects infused with both autologous and homologous C^{14}-5-HTA-labeled platelets has been studied. The half-time of C^{14}-5-HTA in circulating platelets was 5 to 6 days, with less than 20 per cent of the activity present at 8 to 13 days. These values correlate well with reported platelet survival in humans studied by other techniques.

2. If exchange of labeled for nonlabeled 5-HTA in platelets and re-utilization of label are minimal, the disappearance curves indicate an exponential loss of platelets from the circulation compatible with the concept of random disappearance of platelets from the circulation.

3. Calculation of the number of infused labeled platelets that circulate indicates that 50 to 55 per cent was in the peripheral blood at the end of the first day. This value is in reasonable agreement with other estimates of a relatively small total platelet pool.

ACKNOWLEDGMENT

The author wishes to express his appreciation to Mrs. Marie Wasson for excellent technical assistance.

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IRON AND PROTEIN KINETICS STUDIED BY MEANS OF DOUBLY LABELED HUMAN CRYSTALLINE TRANSFERRIN *

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Although proteins isotopically labeled with iodine have been used in tracer studies for some years, interest has been mainly focused on their distribution and degradation in the body. While it is often possible to produce iodoproteins whose physical and chemical properties are not grossly altered, the possible effects of iodination on the functional properties of such substances in a physiologic setting have been less thoroughly investigated. That it is possible to trace label proteins without significantly reducing their biological activities has been demonstrated in the case of insulin (2) and certain anterior pituitary hormones (3, 4).

Transferrin (siderophilin, iron-binding globulin), a β-globulin found in the plasma of man (5, 6) and other animal species (7, 8), is thought to function primarily as a transport vehicle for iron (9). It is remarkably similar in its metal-binding properties to conalbumin, an egg white pseudoglobulin, and, indeed, it has been suggested that the binding sites in the two proteins are similar (10). At neutral or mildly alkaline pH and in the presence of bicarbonate, both proteins firmly bind two ferric ions per molecule, forming colored complexes which have approximately the same absorption maximum (11–14).

It has been shown that the iron-binding capacity of uncomplexed conalbumin is markedly diminished by a variety of chemical procedures which include iodination. This suggests that the specific interaction between metal and protein depends on the structural integrity of the latter (15). However, Azari and Feeney (16, 17) have recently demonstrated that not only is the iron complex of conalbumin more resistant to proteolysis and thermal denaturation than the metal-free protein, but that it is possible to maintain the iron-binding capacity of the former after extensive iodination.

Since both iron and transferrin levels are affected in a variety of disease states, a technique in which both can be followed simultaneously *in vivo* should prove valuable. Elmlinger and coworkers (18) have reported in abstract form on the use of doubly labeled "iron-binding globulin" in human subjects, but few details were given as to the methods of preparation and the distribution of the two labels.

The following study was undertaken to determine if the protein moiety of the iron-transferrin complex could be labeled with iodine without affecting its binding properties. When this was found to be possible *in vitro*, the behavior of the doubly labeled crystalline transferrin was then investigated in eight normal subjects.

METHODS

Three times recrystallized human transferrin was obtained in its iron-free form [1] (19, 20). This material was at least 97 per cent pure, by moving boundary electrophoretic criteria. A similar preparation has been shown to have 0.5 per cent albumin and 0.2 per cent γ-globulin, as determined immunologically (21). Starch gel electrophoresis of this material at pH 8.5, using the method of Smithies (22), yielded a single band corresponding to the transferrin C phenotype (23).

All glassware was acid-washed. Iron was added as $Fe(NH_4)_2(SO_4)_2 \cdot 6\ H_2O$ (with or without Fe^{59}) to solutions of the protein at pH 7.5 (0.1 M Tris,[2] 0.02 M HCO_3^-). After stirring for 1 hour at room temperature, the mixture was allowed to stand overnight at 4 to 5° C to allow sufficient time for oxidation of the ferrous ion

* A preliminary report of this study has appeared in abstract form (1). This work was supported by Training Grant no. 2A-5155 from the National Institute of Arthritis and Metabolic Diseases of the United States Public Health Service to the Boston University School of Medicine.

[1] Kindly provided by the Michigan State Department of Health through the courtesy of the American Red Cross.

[2] Sigma 121; tris(hydroxymethyl)aminomethane.

and full color development. A 20 to 40 per cent excess of iron was used when complete saturation was desired. Optical titrations (6) were carried out in the same buffer by adding varying amounts of iron to equal aliquots of protein. The resultant solutions were checked at intervals to determine if maximal color development had been attained; generally the final readings were not taken for 18 hours.

Iodination was carried out as follows: 8 to 10 per cent solutions of transferrin (iron-saturated or iron-free) were first thoroughly dialyzed against 0.1 M Tris buffer to bring the pH to 8.5 and to remove excess iron. Iodine was added dropwise with constant stirring to samples of the protein kept in an ice bath. The iodinating solutions contained I_2 and KI in a molar ratio of 1:2 or 1:4 plus carrier-free KI^{131}. Because of the presence of bisulfite in commercial KI^{131}, the molarity of the iodine was checked immediately before use by titration against arsenate. After addition of the required amount of the iodine, the mixtures were allowed to stand in the ice bath for 4 to 6 hours and then passed slowly through a Dowex-1 column (Cl^- cycle, buffered at pH 8.5 with 0.01 M Tris). This removed 98 to 99 per cent of the excess I^-. When extinction coefficients were to be determined, the solutions were then dialyzed thoroughly against several changes of 0.02 M Tris, 0.1 M NaCl, pH 7.5 buffer. Dry weights (determined in duplicate) were determined by lyophilization of duplicate aliquots in a vacuum oven at 65° C until constant weight was achieved. The weight of the salt in the final dialysate was subtracted to give the net weight of protein. No Donnan correction was made. The optical density was read against a dialysate blank.

For purposes of stoichiometry the reaction was presumed to proceed as follows: $I_2^- + R \rightarrow RI + 2\ I^-$.

It should be noted that when iodine (2 equivalents per mole) and iodide are present in the iodinating mixture in a molar ratio of 1:2, a maximum of 25 per cent of the added radioactivity will be bound if the above reaction goes to completion, assuming that the labeled iodide is in ready equilibrium with the stable triiodide ion. Thus, the iodine content of the transferrin may be calculated from the radioactivity of the protein per unit weight, with reference to the specific activity of the iodinating solution expressed in terms of counts per equivalent of total iodine plus iodide. It was in fact demonstrated that the specific activity of the labeled transferrin was within 6 per cent of that of the iodinating mixture by means of direct chemical determinations of each in two experiments.[2] In these calculations the molecular weight of transferrin was taken as 86,000. The yield, as given in the results, compares the actual iodine content per mole of protein with that of the theoretical maximum under a given set of conditions.

When prepared for injection, transferrin was initially saturated with stable iron and, after iodination as above with a total of 2 to 3 mc I^{131}, the iron was removed by lowering the pH to 5.5 to 5.6 with an acetate-EDTA[4] buffer (19). Decolorization of the complex was complete in 1 hour; the solutions were then dialyzed to bring the pH back to 7.5 and to remove the EDTA. Fe^{59} was then added to the desired level of saturation. After further dialysis against 0.02 M $NaHCO_3$, 0.1 M NaCl (pH 7.4 to 7.6), the solution was passed through a Millipore filter[5] (pore size 0.3 μ) into sterile containers. The final concentration of protein was 2.5 to 4.0 g per 100 ml. It contained 12 to 25 μc of I^{131}, and 1 to 2 μc of Fe^{59} per ml. Less than 0.1 per cent of the iodine radioactivity was unbound as determined by trichloroacetic acid precipitation.

Processing took 3 days and the material was administered within 5 days of the initial addition of I^{131}. It was calculated (24) that no more than 3,000 rad was absorbed by the transferrin prior to injection.

Analytic ultracentrifugal patterns were obtained in a model E Spinco ultracentrifuge.[6] The human transferrin immune serum used was derived from rabbits[7] and was found to produce a single band of precipitation against serum and crystalline transferrin in Ouchterlony (25) plates. Quantitative precipitin curves in the zone of antibody excess were constructed by the method of Gitlin (26).

Chromatograms were done on Whatman no. 1 paper using the salt-saturated solvent (A) described by McMenamy, Lund and Oncley (27). Digests were prepared in 4 M $Ba(OH)_2$ in sealed tubes at 105° C. The papers were stained with either Pauly's reagent or isatin, as described by the same authors. Monoiodotyrosine (Rf, 0.41) and diiodotyrosine (Rf, 0.19) reacted with both these stains, although less well than tyrosine itself. In addition to visual estimation against standards, the iodinated protein chromatograms were scanned in a strip counter or radioautographed.

The volunteers used in the *in vivo* studies were all healthy white males, 25 to 43 years of age. All received 10 drops of Lugol's solution daily. The doubly labeled transferrin was delivered intravenously from calibrated syringes from which standards were also prepared. The total amount of protein and iron injected did not exceed 70 mg and 90 μg, respectively (about 2 to 3 per cent of the total plasma content of each). Each subject received 30 to 50 μc of I^{131} and 3 to 4 μc of Fe^{59}.

Six to 7 ml of blood was drawn at 10- to 30-minute intervals for the first 3 hours and approximately 6 hours after injection and the plasma immediately separated. Daily samples were taken thereafter for 18 to 21 days and, in addition to plasma, aliquots of whole blood, hemolyzed with saponin, were saved for counting. Serum iron and total iron-binding capacity (TIBC) were determined on samples drawn immediately before injection of transfer-

[2] These determinations were performed by the Graham Laboratories, Brookline, Mass.

[4] Ethylenediamine tetraacetic acid (disodium salt).

[5] Millipore Corporation, Bedford, Mass.

[6] Kindly performed through the courtesy of Dr. J. L. Oncley and Mr. Charles Gordon, Dept. of Biological Chemistry, Harvard Medical School.

[7] Behringwerke, Marburg-Lahn, West Germany.

rin and about 2 weeks later. The method of Peters, Giovanniello, Apt and Ross (28–30) was used for both.

Radioactivity was measured in a sodium iodide scintillation well counter. Simultaneous Fe^{59} and I^{131} content was determined using an adaptation of the method of Hine and associates (31) in which two scalers, set at different discrimination levels (200 and 700 Kev), were employed. The sample counts are then compared to pure Fe^{59} and I^{131} under identical conditions. By means of the comparative counting sensitivities of the scalers, simultaneous equations may be derived which define the relative proportions and absolute amounts of the two isotopes in the mixed sample. This method yields a higher counting rate than would be obtained by using a narrow-window pulse-height analyzer. Net counting rate on the scaler with the lower discriminator setting was at least three times background, and sufficient counting time was employed to give an over-all counting error of not more than 3 to 4 per cent.

Body counting was performed with the apparatus described by Hine, Jagger and Burrows (32). The recording system was as described above, except that a correction was made for the difference in geometry between standards and the body.

The terms and methods of calculation used for iron kinetics are those of Huff and co-workers (33). Venous hematocrit was corrected by a factor of 0.92 in calculating red cell mass. Plasma volume was determined on the basis of the dilution of I^{131} as extrapolated to zero time. Transferrin concentration of plasma in milligrams per 100 ml was calculated as TIBC (μg per 100 ml)/1.30. Immunologic determinations of transferrin concentration agreed closely with the above.

RESULTS

A. Chemical studies. The addition of iodine to both iron-free and iron-saturated transferrin was practically quantitative, 89 to 98 per cent of the theoretical yields being obtained. This is apparently due to the large number of tyrosine residues present (about 30 per mole on the basis of chromatographic estimation) and the absence of reduced sulfhydryl groups in the transferrin molecule (34).

The original transferrin, fully saturated with iron, had a broad visible absorption maximum with a peak at 465 mμ where the extinction coefficient ($E^{1\%}_{465}$) was 0.63.[8] Optical titration at this wave length indicated a binding capacity of 1.30 Fe per mg transferrin, equivalent to a molecular weight of 86,000 for the protein. The same value was obtained by means of the specific activity of Fe^{59} in samples that were first passed through a cation-exchange column, which removes traces of nonchromogenically bound iron.

[8] When corrected for the absorption of the iron-free transferrin at this wave length, as is done by Inman and colleagues (20), $E^{1\%}_{465}$ is 0.60.

TABLE I

Effect of iodination of iron-saturated transferrin on the visible extinction and binding capacity *

Moles I/ mole transferrin	$E^{1\%}_{465}$	Binding capacity (μg Fe/mg) transferrin
0.0	0.63	1.30
0.9	0.63	1.31
2.0	0.63	1.29
5.7	0.63	1.33
9.2	0.62	1.29
14.0	0.61	1.28

* All of the above were iodinated as the iron complex. Values were obtained after dialysis and treatment with cation-exchange resin, pH 7.5.

The effect of iodination on the visible extinction of transferrin and its iron-binding capacity is shown in Table I. Only a slight change was noted when up to 14 atoms of iodine per mole of protein were incorporated into the iron-complexed protein. In contrast, a moderate but progressive decrease in $E^{1\%}_{465}$ was noted when iron-free transferrin was iodinated and subsequently saturated with iron (Table II).

The ultraviolet spectrum in 0.1 N NaOH was little changed in the region of 279 mμ, but increasing absorption was noted in the region of 308 to 312 mμ as the iodine content of the transferrin was increased. Although this appeared to indicate the presence of diiodotyrosine, which has its absorption maximum at 311 mμ, chromatographic analysis of the iodoproteins indicated that all of the radioactivity moved as monoiodotyrosine. Evidently the absorption maximum of monoiodoty-

TABLE II

*Effect of iodination of iron-free transferrin on the visible extinction after subsequent iron saturation**

Moles I/ mole transferrin	$E^{1\%}_{465}$
0.0	0.63
0.7	0.59
1.5	0.58
5.1	0.54
8.5	0.51

* All of the above were iodinated in the iron-free state. The extinctions were obtained after addition of excess iron, followed by dialysis. Binding capacity was not determined as such in this series.

rosine, which is 305 mμ in basic solutions, is increased somewhat in the peptide chain.

When lightly iodinated transferrin (0.9 atoms iodine per mole) saturated with Fe^{59} was dialyzed at room temperature for 48 hours against a large volume of human serum, no radioiodide appeared in the dialysate. The great stability of the iron protein complex was indicated by the fact that only 9 per cent of the theoretical amount of iron that could be bound by the transferrin in the serum crossed the membrane in the 48 hours.

Iron was removed from this preparation by lowering the pH to 5.6 in the presence of EDTA. After EDTA was eliminated by dialysis and the pH raised to 7.5, the binding capacity was unchanged. $E^{1\%}_{279}$ of the iron-free material was 11.1 and that of the iron-saturated protein 13.7, which were the same as the values of the uniodinated transferrin.

Ultracentrifugation of the lightly iodinated transferrin in 0.15 M NaCl, 0.01 M Tris, pH 7.5, gave patterns which were identical with that of the original protein. Sedimentation of a 1 per cent solution of iron-saturated transferrin at 25° C was 5.1S (Figure 1). Immunologic studies on this material revealed no obvious alteration in either the precipitation patterns produced in Ouchterlony plates or in the quantitative precipitin curves as compared with normal human serum and untreated crystalline transferrin.

B. In vivo studies. Four separate preparations of iodinated transferrin, each doubly labeled, were used in these studies: I) 0.4 atoms iodine per mole and 1.32 μg Fe per mg transferrin (100 per cent saturation), II) 0.8 atoms iodine per mole and 1.3 μg Fe per mg transferrin (100 per cent saturation), III) 0.6 atoms iodine per mole and 0.57 μg Fe per mg transferrin (42 per cent saturation), IV) 0.6 atoms iodine per mole and 0.65 μg Fe per mg transferrin (50 per cent saturation). All were prepared from the same lot of crystalline transferrin.

Ferrokinetic data in the eight subjects are summarized in Table III. Since relatively small amounts of Fe^{59} were injected, plasma clearance could be accurately followed for only 3 to 6 hours

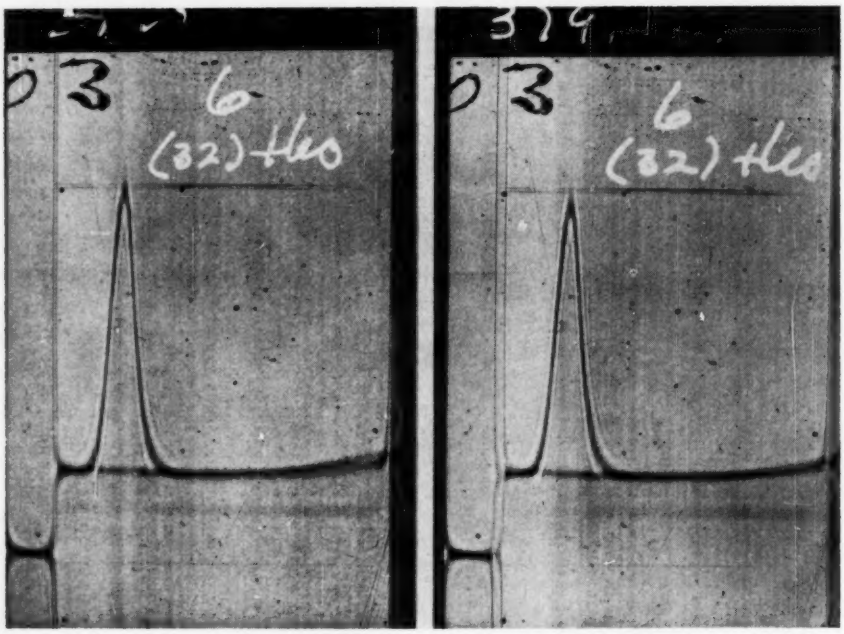

FIG. 1. ULTRACENTRIFUGAL PATTERNS OF CRYSTALLINE TRANSFERRIN, 0.99 G PER 100 ML (LEFT) AND OF IODINATED TRANSFERRIN WITH 0.9 ATOMS IODINE PER MOLE PROTEIN, 0.96 G PER 100 ML (RIGHT). Both preparations saturated with iron and dialyzed against a pH 7.5, 0.15 M NaCl, 0.01 M Tris buffer, 59,780 rpm; bar angle + 60°, time 32 minutes, temperature 25° C.

TABLE III

Ferrokinetics of doubly labeled transferrin

Subject	Preparation	Venous hct.	Plasma Fe	$T_{\frac{1}{2}}Fe$*	Plasma Fe turnover	RBC Fe utilization	RCB Fe turnover
		%	*μg/100 ml*	*hrs*	*mg/kg/day*	*% dose*	*mg/kg/day*
H.L.	I	45	94	1.05	0.67	86	0.58
R.M.	I	47	124	1.32	0.69	85	0.56
A.D.	II	45	163	2.48	0.48	75	0.36
H.W.	II	47	86	0.98	0.63	92	0.59
E.M.	III	46	130	1.63	0.58	81	0.48
P.R.	III	45	146	2.00	0.52	81	0.42
H.C.	III	44	123	2.00	0.49	85	0.42
S.M.	IV	48	89	1.93	0.34	87	0.30

* Half-life of plasma iron disappearance.

after injection. During this interval the iron disappeared at what seemed to be a single exponential rate. With the exception of A.D., whose serum iron was at the upper limit of normal, it appeared that the clearance was more rapid in those subjects given transferrin fully saturated with iron. Variation in serum iron levels makes direct comparison difficult, since the calculated plasma iron turnover values diminished the apparent differences, although the three highest values for turnover were found in those subjects given preparations I and II. However, all values were within the limits previously established in this laboratory (35, 36) [9] and in others (37, 38) when whole plasma has been used to bind the radioiron.

Red cell utilization of iron was maximal on the eighth to tenth day after injection, at which time 75 to 92 per cent of the Fe^{59} was in the circulating red cell mass. This is again within the range found when plasma-bound iron is injected.[9]

Protein kinetics are summarized in Table IV. The initial rate of disappearance of transferrin from the plasma (over the first 3 to 6 hours) did not exceed 5.7 per cent per hour, i.e., the half-life

[9] The values obtained in this laboratory in normal subjects using whole plasma to bind radioiron have been: *a*) plasma iron half-life ($T_{\frac{1}{2}}Fe$), 1.0 to 2.4 hours; *b*) plasma iron turnover, 0.25 to 0.70 mg per kg per day; *c*) maximal red blood cell Fe utilization, 75 to 95 per cent of dose injected.

TABLE IV

Protein kinetics of doubly labeled transferrin

Subject	Wt	Plasma transferrin	Plasma volume	$T_{\frac{1}{2}i}$*	Total transferrin pool	Extravascular fraction of transferrin pool	$T_{\frac{1}{2}p}$†	Transferrin degraded per day
	kg	*mg%*	*ml/kg*	*hrs*	*g/kg*		*days*	*g/kg*
H.L.	45.6	255	45	21	0.27	0.57	8.0	0.023
R.M.	71.0	256	44	16	0.30	0.62	7.0	0.030
A.D.	90.5	205	44	18	0.20	0.56	7.5	0.019
H.W.	53.6	222	43	14	0.25	0.62	7.8	0.022
E.M.	77.5	213	44	23	0.23	0.58	6.7	0.024
P.R.	60.8	236	48	15	0.29	0.61	7.5	0.027
H.C.	55.0	215	47	15	0.25	0.59	7.7	0.023
S.M.	70.5	246	44	12	0.27	0.59	8.4	0.022

* $T_{\frac{1}{2}i}$ = half-life of initial plasma transferrin disappearance (diffusion).
† $T_{\frac{1}{2}p}$ = plasma half-life of transferrin after equilibrium was achieved (degradation).

of this initial disappearance ($T_{\frac{1}{2}}$) was greater than 12 hours. This is about the same rate at which albumin initially diffuses from the plasma (39). No significant difference was noted in the four preparations used. Plasma volumes obtained by extrapolating iodine counts to zero time were likewise similar to those obtained by others when radioalbumin was used (40). Indeed, in two subjects Evans blue plasma volume determinations were within 8 per cent of the isotope dilution volumes.

In all subjects, zero time I^{131} and Fe^{59} counts, expressed as the fraction of the respective isotope injected, agreed quite closely (Figure 2A). Graphical analysis of curves constructed from plasma transferrin disappearance over the ensuing 3 days (Figure 2B) indicated that the initial rate of disappearance of the two isotopes was identical.

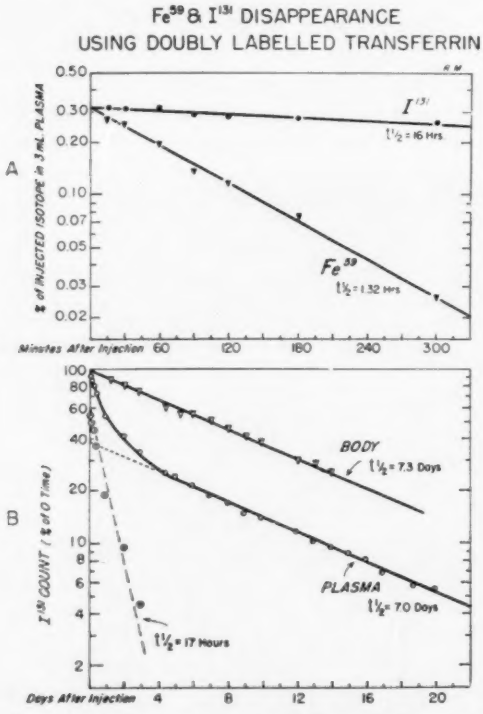

FIG. 2. A) DISAPPEARANCE OF Fe^{59} AND I^{131} FROM THE PLASMA IN SUBJECT R.M. OVER THE FIRST 5 HOURS AFTER INJECTION, PLOTTED ON SEMILOGARITHMIC SCALE. Abscissa units are in minutes. B) DISAPPEARANCE OF I^{131} FROM THE PLASMA AND "BODY" IN R.M. Abscissa units are in days. The dashed line in the lower left-hand corner represents the initial component of plasma disappearance as extrapolated from the linear portion of the plasma curve.

fusion did not vary significantly and corresponded to the rate observed over the first few hours. Although this apparently indicates that plasma transferrin interchanges with a single extravascular compartment (41), the presence of additional small or very slowly turned over compartments cannot be excluded from the available data. Equilibrium of the tracer in the plasma with such a compartment or compartments was achieved in 4 to 5 days. Thereafter the protein disappeared at a single exponential rate with a half-life of 6.7 to 8.4 days (the half-life obtained from Days 5 to 12 did not differ significantly from the half-life obtained by including subsequent points). Extrapolation of this linear portion of the curve to zero time indicated that 56 to 62 per cent of the total exchangeable transferrin was extravascular.

Body counting of radioiodine (Figure 2B) in two subjects gave disappearance rates which were in fair agreement with that obtained from the linear phase of the plasma curves (in H.L. plasma and body half-lives were 8.0 and 8.7 days and in R.M. 7.0 and 7.3, respectively). No evidence of localization of radioiodide was found in the region of the thyroid. Previous observations using radioiodinated albumin have indicated that body-counting disappearance curves closely paralleled those derived from urinary and fecal excretion data (42). It is therefore felt that the plasma data in this study gave a good estimate of the actual degradation rate.

The total exchangeable pool of transferrin ranged from 0.23 to 0.30 g per kg body weight. On this basis 0.022 to 0.030 g was degraded per day per kg. Since transferrin levels were reasonably stable (levels did not vary by more than 10 per cent during the period of study), a steady state may be assumed and the same figures would apply to the synthetic rate.

DISCUSSION

The great avidity of transferrin for iron appears to account for its primary function as a regulator of the metabolism of the metal in the body. The concentration of ionic iron in the presence of transferrin is exceedingly small provided the binding capacity has not been exceeded. The stability constant of the complex has been estimated as 10^{29} (43). Recent evidence has suggested that it may

play an important part in the control of absorption of iron from the gut (44). Furthermore, the interaction of the protein at specific cellular sites may determine the distribution and chemical form of the iron incorporated into such tissues as marrow and liver (45, 46). The studies presented herein indicate that the protein moiety of the transferrin-Fe complex can be labeled with radioiodine without changing its binding properties *in vitro*. Gross physical and immunologic characteristics were likewise unchanged. It was felt, therefore, that such a substance could be validly employed as a tracer *in vivo* when both iron and protein kinetics were to be followed.

Dialysis studies indicated that there is a very slow interchange of iron with unsaturated transferrin once the metal is bound within a given molecule. A similar observation has been made recently by Turnbull and Giblett (47) who have employed electrophoretically separable variants of transferrin. It is, therefore, a reasonable assumption that the two isotopes remain on the same transferrin molecule until the iron is removed, there being essentially no interchange of the latter with indigenous protein during the first few hours after injection.

Bothwell, Hurtado, Donohue and Finch (38) found that iron disappearance was significantly slower in subjects given radioiron bound to fraction IV-7 of Cohn as compared with simultaneously determined clearances using iron bound to plasma. The crystalline transferrin used in the present study is derived from fraction IV-4, the immediate source of fraction IV-7 (20). However, the plasma clearance and red cell utilization in the eight subjects studied was within the relatively wide range found by these workers and others (35–37) when plasma-bound iron is injected. It is realized that comparison with previous data of this sort is somewhat tenuous in view of the variability of these values, not only among different subjects but for the same individual at different times. The use of a double-iron isotope technique employing Fe^{55} and Fe^{59} (38, 47), which might have allowed the direct assessment of any differences in clearance, was precluded by the lack of suitable equipment and the desire to minimize radiation dosage. While it cannot therefore be stated with certainty that there was no alteration in the unloading and normal distribution of iron *in vivo*, the results suggest that these characteristics are not grossly affected by the methods employed in the isolation and iodination of transferrin.

The observation that transferrin disappears from the plasma compartment much more slowly than the rate at which iron is cleared is similar to the findings of Elmlinger and associates (18), and is consistent with the hypothesis of Laurell that iron is dissociated from transferrin prior to the passage of the protein through the capillaries (9). However, the latter author stated that the process by which this dissociation occurs involves a simple equilibrium between intra- and extravascular ionic iron. In view of the strength of binding, as noted above, it is difficult to see how such a process can account for the rapidity with which iron disappears from the plasma.

Jandl, Inman, Simmons and Allen (45) have indicated that transferrin in some manner preferentially directs iron into hemoglobin-synthesizing reticulocytes in the presence of mature red cells. They suggested that this depends on a specific interaction of transferrin with the surface of reticulocytes. Recent studies employing a preparation of transferrin identical with those used in this report appear to confirm this hypothesis (48).

If such adsorption occurs on erythropoietic cells in general, another possible explanation for the discrepant rates of iron and protein disappearance may be inferred. Bone marrow sinusoids, which are intimately dispersed throughout erythropoietic tissue, appear to be lined by multifunctional reticular cells, to lack a definite basement membrane, and to be frequently discontinuous (49, 50). Furthermore, the marrow does not contain separate lymphatic vessels, the sinusoids presumably subserving this function. The circulation is therefore "fundamentally open in character" (49). Thus, transferrin may be directly absorbed from and eluted into the functional confines of the vascular compartment. Since the turnover rates of adsorbed transferrin in reticuloyctes have been found to be quite rapid (48), probably only a small fraction of the transferrin molecules present in this compartment is adsorbed (i.e., sequestered) at any given time, at least in normal subjects. Therefore, no great effect on plasma volume would be noted when determined by dilution of the protein tracer. The clearance of iron may

thus be a function both of the rate of turnover of the adsorbed Fe-transferrin complex and the rate of extraction of the metal therefrom by the developing red cells. Some evidence that these two rate processes are interrelated has been obtained from the *in vitro* studies cited above (48). In any event, the extravascular migration of iron in the complexed or ionic state need not be involved.

More than 50 per cent of the exchangeable pool of transferrin was found to be extravascular. Part of this extravascular protein can be found within the cells of various tissues (51). The relatively rapid changes seen in the iron-binding capacity of plasma, which occur in infection, may be a result of redistribution of the transferrin pool. In this regard, Cartwright, Gubler and Wintrobe (52) have found that the reticuloendothelial blockade produced by thorium dioxide inhibits the fall in transferrin level usually noted in infected dogs. Similarly, the increase in plasma transferrin found in iron-deficient states may represent shifts in the opposite direction. The extremely rapid changes observed by Mitchell and co-workers (53) when iron is given in such a situation can only be explained on the basis of redistribution of the transferrin. As indicated by these authors, this may well be due to an increase in transferrin adsorption to cellular sites.

The turnover of transferrin as obtained in this study lay between that observed for albumin (54) on the one hand, and the lipoproteins (55) on the other. Gitlin, Janeway and Farr (56), who also used iodine-labeled crystalline transferrin, found that the metabolic half-life was 12 days in one normal child.

Not only may the procedures employed in obtaining fractions for labeling affect the degradation rates of the protein, but other factors, such as the extent of iodination (57) and the degree of absorption of radiation (54, 58), affect its behavior in the body. Although care was taken to minimize the latter factors, it is impossible to state how closely the turnover of the transferrin used in this study corresponds to that of the native protein. However, the fact that several preparations disappeared at an apparently uniform and reproducible rate over 3 weeks, during which time more than 75 per cent of the labeled transferrin had been degraded, suggests that no significant degree of catabolic heterogeneity had been produced.

SUMMARY

1. Iodine may be incorporated in small amounts into transferrin in its iron-complexed state without materially affecting its binding capacity and immunologic properties *in vitro*.

2. Iron and protein kinetics were followed in eight subjects after the administration of human crystalline transferrin labeled with both Fe^{59} and I^{131}.

3. Iron clearance and red cell utilization were within the limits found in previous studies which have used radioiron bound to plasma. Initial transferrin disappearance from plasma was at least six times slower than the rate at which iron was cleared.

4. The total exchangeable pool of transferrin was determined as 0.2 to 0.3 g per kg body weight, of which 56 to 62 per cent was extravascular.

5. The metabolic half-life of four separate preparations of iodinated transferrin varied from 6.7 to 8.4 days.

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CORRECTION

In the paper "The Incorporation of Plasma Free Fatty Acids into Plasma Triglycerides in Man," by S. J. Friedberg, R. F. Klein, D. L. Trout, M. D. Bogdonoff and E. H. Estes, Jr., 1961, **40**, 1852 (October), the following errors occurred.

For:	read:
$z=x-y$	$z=x+y$
$z=x-k\int_{t_0}^{t_1} x\,dt$	$z=x+k\int_{t_0}^{t_1} x\,dt$
$F=z/M=\left[x-k\int_{t_0}^{t_1} x\,dt\right]\Big/M$	$F=z/M=\left[x+k\int_{t_0}^{t_1} x\,dt\right]\Big/M$
$Fa=a/M\left[x-k\int_{t_0}^{t_1} x\,dt\right]$	$Fa=a/M\left[x+k\int_{t_0}^{t_1} x\,dt\right]$
Plasma TGFA content	Plasma TGFA content
$=a/Mk\left[x-k\int_{t_0}^{t_1} x\,dt\right]$	$=a/Mk\left[x+k\int_{t_0}^{t_1} x\,dt\right]$
$=a/M\left[x/k-\int_{t_0}^{t_1} x\,dt\right]$	$=a/M\left[x/k+\int_{t_0}^{t_1} x\,dt\right]$

A TEST OF CONNECTIVE TISSUE STATE AND REACTIVITY IN COLLAGEN DISEASES *

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In the group of conditions known as "collagen diseases" there are often morphological changes in discrete areas of the connective tissue. For example, fibrinoid degeneration of the collagen fibers and changes in the ground substance may be seen in the subcutaneous nodules of rheumatoid arthritis and in the skin lesions of lupus erythematosus. These lesions are not well understood. The more generalized alterations in the connective tissue are even more difficult to demonstrate and remain poorly defined. Using an *in vivo* electrometric method, we have previously determined certain physicochemical properties of normal mammalian connective tissue which are related to its amphoteric colloidal structure (1–5). Since these properties may be altered in pathological states, we have adapted this method to study some of the fundamental connective tissue changes in collagen diseases of humans.

Based upon histochemical, physicochemical and morphological studies, a unified concept of the organization of the extracellular part of normal connective tissue has been developed (6–11). The ground substance has been characterized physicochemically as a two-phase system containing heterogeneous (soluble and insoluble) colloids, electrolytes and water. A water-rich phase containing the soluble components is considered to be in equilibrium with a colloid-rich phase. The existence of these phases at a submicroscopic level has been demonstrated with the electron microscope. The water-rich phase consists of vacuoles with diameters ranging from 600 to 1,200 Ångstrom units (7–10). These vacuoles are enclosed by a much denser colloid-rich phase. Under physiological conditions the amphoteric colloidal matrix bears a net negative charge; this is distributed between the two phases. The colloidal charge governs the distribution of mobile cations and anions through thermodynamic and electrostatic equilibrium and through the chemical binding of ions to the matrix. Electrolyte concentrations and water content in connective tissues are thus determined by the colloidal structure (1–5).

Collagen, glycoproteins and mucopolysaccharides, including chondroitin sulfate and hyaluronic acid, have been conceived to be constituents of a complex coacervate in the matrix of dermal connective tissue (12). The isoelectric point of the matrix ranges from about pH 2.2 to 2.5 depending on its composition. The low isoelectric point is a result of the acidic character of substances such as chondroitin sulfate and other mucopolysaccharides. The density of negative charge of the colloidal matrix is related to the number of titratable groups which bind hydrogen ions between pH 7.4 (physiological) and the isoelectric point (pH 2.2 to 2.5). This depends on the compostion of the matrix with respect to all the colloidal components. That these form complex insoluble aggregates may be inferred from the difficulties usually encountered in attempts to isolate the colloidal components in pure form.

When the enzyme hyaluronidase is injected into the dermis, hyaluronic acid and some of the chondroitin sulfate are disaggregated. This leads to measurable changes in spreading properties and to a lowering of the negative charge density (13–16). The effect may be explained by: *1*) removal of negatively charged components, such as hyaluronic acid and chondroitin sulfate A, accompanied by *2*) redistribution of water and electrolytes within the region of spreading. By the action of the enzyme, the normal balance between soluble and insoluble colloidal components is disturbed, and the remaining insoluble colloids are characterized by

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a low density of negative charge. In animal experiments, the effect of hyaluronidase applied subcutaneously is to lower the negative colloidal charge to less than half the initial level (14). The initial state, as indicated by the negative charge density, may be compared with a hyaluronidase-modified state produced during a standard time interval. After the colloidal charge density becomes minimal the subsequent recovery curve, observed electrometrically, illustrates the functional state of the connective tissue (14–16). The serial electrometric evaluation of colloidal charge offers a means of comparing normal and pathological tissues and the effects of various treatments.

THEORETICAL

At an equilibrium boundary between a charged colloidal surface and an isotonic saline solution there is a difference of potential which depends on the density of colloidal charge and on the composition of the applied electrolyte solution (5, 17, 18). This is the normal Donnan potential, E_D, which is related to the Donnan ratio, r, by the formula:

$$E_D = \frac{2.303\ RT}{96{,}500} \log_{10} r \qquad [1]$$

where 2.303 is the conversion factor between common and natural logarithms, R is the gas constant in joules per degree, T is the absolute temperature, and 96,500 is the value of the Faraday constant in coulombs. E_D is usually not determined directly in tissues; it is measured indirectly by the potential at the surface after substitution of 0.015 M NaCl at the boundary (5, 17). This yields a displaced Donnan potential, E_D', related to the following quantities: the Donnan potential, the Donnan ratio, the fractions of current transported by anions and cations, and the density of negative colloidal charge, x (5, 17, 18). Thus

$$E_D' - E_D = \frac{2.303\ RT}{96{,}500} (n_1' - n_2') \qquad [2]$$

where n_1' and n_2' denote the fractions of current carried respectively by cations and anions in the colloidal phase. The difference is related to the Donnan ratio, r, by the formula:

$$n_1' - n_2' = \frac{r^2 - 1.52}{r^2 + 1.52} \qquad [3]$$

where 1.52 is the ratio of the ionic conductance of chloride to that of sodium (5, 17). The density of negative colloidal charge, x, in equivalents (Eq) per kilogram water is related to r by the formula

$$x = 0.15\ (r - 1/r) \qquad [4]$$

where $r = \frac{(Na^+)_2}{(Na^+)_1} = \frac{(Cl^-)_1}{(Cl^-)_2}$. The subscripts 1 and 2 refer to the solution phase (0.15 M), and the colloidal phase, respectively. Graphical methods have been described to estimate x, the colloidal charge, from $(E_D' - E_D)$ at any pH (5). At pH 7.4 and 37° C, for values of x less than about 0.06 Eq per kg water, there is a simple linear relation:

$$E_d = -12.3 + 206\ x \qquad [5]$$

where E_d denotes the difference $(E_D' - E_D)$, and is termed the "dilution potential." On the basis of this method, titration curves of numerous colloidal surfaces have been measured. These include dermis, cartilage and epidermis *in vivo*, and wool surfaces *in vitro* (5, 17, 18).

MATERIAL AND METHODS

The connective tissue function test was performed in 10 healthy persons and in 14 untreated patients with various connective tissue diseases. The age and sex distributions of the two groups are given in Table I. The following pathological conditions were studied: rheumatoid arthritis (3 cases), systemic lupus erythematosus (3 cases), and scleroderma (8 cases). In addition, the alteration in connective tissue was also investigated in 3 patients with Cushing's syndrome. A number of patients in each group was retested after periods of treatment.

Displaced Donnan potentials in dermal connective tissue were measured by a modification of the method previously described for animal studies (5, 14–16). Details of the method are as follows: a 25 gauge platinum needle with a glass hub was inserted into the dermis on the anterior surface of the right forearm.[1] The reference

[1] In human subjects the use of a platinum needle is preferable to surgical exposure of the dermis and direct application of the solutions. In experiments with rats and rabbits the dilution potential in the dermis has been found to be about −2 mv after direct exposure of the tissue to the dilute solution (5). The same result has been found in a series of animals and humans using the platinum needle technique. In both series of experiments the calculated value of x is about 0.050 Eq. Since the platinum needle is filled with saline solution, the metal is at constant potential. Contact with the solution is made by means of saturated KCl, and there is no

TABLE I

Electrometric results in normal subjects and in patients with connective tissue diseases *

Condition	Male	Female	Age range	Baseline dilution potential (E_d^0)	Minimum dilution potential (E_d)†	k	Half-recovery time
			yrs	*mv*	*mv*		*min*
Normal subjects	5	5	22–76	−1.9 ± 0.7	− 7.9 ± 0.8	0.0021 ±0.0006	156 ± 40
Rheumatoid arthritis	0	3	40–47	−9.1 (−8.0 to −10.1)	−12.3 (−12.0 to −12.5)	0.0000	No recovery
Lupus erythematosus	1	2	14–45	−4.9 −(4.4 to − 5.2)	−11.1 (− 9.9 to −11.8)	0.0000	No recovery
Scleroderma	0	8	28–63	−9.6 ± 0.8	−12.1 ± 0.4	0.0000	No recovery
Cushing's syndrome	1	2	20–35	−5.7 (−5.5 to − 6.0)	−11.3 (−10.8 to −11.7)	0.0047	65

* Mean values (± standard deviations where stated). Dilution potentials (E_d) can be converted to negative charge density (x) by the formula $E_d = -12.3 + 206\ x$.

† The lowest value of E_d obtained after the injection of hyaluronidase into the dermis.

junction was made by immersing several fingers of the left hand in a beaker containing 0.15 M NaCl (Figure 1). The lumen of the platinum needle was filled with 0.1 to 0.2 ml of sterile buffered (pH 7.4) 0.15 M NaCl (solution I), making contact with the dermal connective tissue. The solution was prepared by mixing 10 ml of 0.075 M Na_2HPO_4 with 1 ml 0.15 M NaH_2PO_4, and diluting to 110 ml with 0.15 M NaCl. The solutions at the experimental junction and in the reference beaker were each connected to saturated KCl-calomel half-cells by means of saturated KCl salt bridges. The ends of the connecting tubes were sealed with small tapered glass tubes filled with saturated KCl in solidified agar. A similar method of studying epidermis has been described (17). Potentials were measured with a Leeds-Northrup type K-2 potentiometer and a galvanometer sensitive to 0.1 mv. The circuit is represented as follows:

$$Hg \mid Hg_2Cl_2 \mid KCl\ (sat.) \mid \text{solution I or II} \mid \text{dermis} \mid \text{finger} \mid \text{solution I} \mid KCl\ (sat.) \mid Hg_2Cl_2 \mid Hg$$

An initial potential (E_1) between the experimental (forearm dermis) and reference (finger) sites was determined with solution I (buffered isotonic NaCl) at the experimental junction. The solution was then aspirated and replaced by 0.1 to 0.2 ml of a 1/10 dilution (0.015 M NaCl, solution II). The displaced potential (E_2) was measured. The initial potential was then re-established with solution I at the experimental site. Each value was based on the mean of two consecutive readings taken over a period of 2 to 3 minutes. The mean value of ($E_2 - E_1$) measured before the action of hyaluronidase is denoted as the initial dilution potential, E_d^0. The potentials E_1 and E_2 were generally quite constant and reproducible. The values of E_1 preceding the measurement of E_2 generally agreed to within 0.5 mv. Two values of E_1 following the measurement of E_2 agreed equally well (Table II). Standard deviations of four readings were

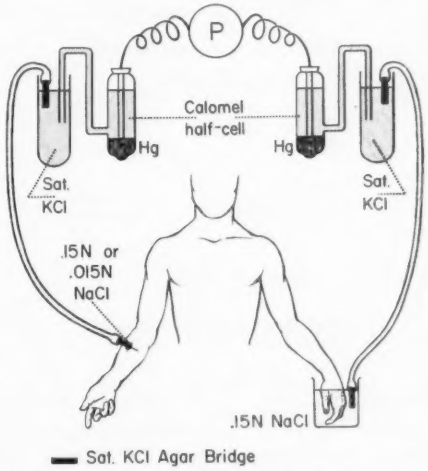

FIG. 1. DIAGRAM OF THE APPARATUS USED FOR THE ELECTROMETRIC DETERMINATION OF DISPLACED DONNAN POTENTIALS IN THE DERMIS.

metallic contact with the platinum. For these reasons the flow of current is entirely through the solutions. The system may therefore be treated by the same theoretical and mathematical methods that have been applied to wool, epidermis, and to surgically exposed tissue surfaces (5, 17, 18). In most subjects, the magnitude of the potential E_1 was about 40 to 50 mv, with the experimental (forearm) junction positive with respect to the reference (finger) junction. The sign of the potential always refers to the experimental site.

about 0.3 to 0.5 mv compared with E_d values of about -2 mv (normal), and -8 mv (normal plus hyaluronidase).

Immediately after determination of E_d, 15 turbidity reducing units of testicular hyaluronidase (Wyeth), freshly dissolved in 0.2 ml of buffered saline, pH 7.4, were injected into the test site with a 29 gauge needle inserted into the lumen of the platinum needle. Dilution potentials were measured at intervals of 15 and 30 minutes, and at 1, 2, 3 and 4 hours after injection, unless half-recovery occurred more rapidly. Control experiments were made over similar time intervals using a 0.15 M NaCl solution which contained no enzyme. In healthy subjects, these showed no appreciable change of potential over periods of 1 to 2 hours. From the dilution potential, E_d, the negative colloidal charge density, x, of the dermal connective tissue may be calculated according to Equation 5. The values of x may thus be followed in relation to time after the action of the enzyme.

RESULTS

Normal subjects. In a typical experiment on healthy young adults, the initial value of the dilution potential was about -2 mv. This represents a colloidal charge of about 0.05 Eq per kg tissue water, corresponding closely to that found for normal dermal connective tissue of rats and rabbits (5). After the action of testicular hyaluronidase the dilution potential fell to about -8 mv on the average, corresponding to a negative colloidal charge of about 0.02 Eq, about 40 per cent of the normal level. This level was observed after 15 to 30 minutes' exposure to the enzyme. Readings of E_1 and E_2 before and after hyaluronidase are given for a healthy subject and a patient (Table II). After the period of exposure, serial readings were made, and were found to increase during half-hour or 1-hour intervals. Half-recovery for healthy subjects was found to occur between 2 and 3 hours after the minimal potential of -8 mv had been observed. For a subject showing an initial value of colloidal charge of 0.05 Eq and a minimal value of 0.02 Eq, half-recovery would correspond

TABLE II

Initial and displaced potentials of a healthy subject compared with those of scleroderma patient*

Subject, Sex, Age	Condition		Initial potential E_1 Isotonic, pH 7.4	Displaced potential E_2 0.015 M NaCl	Dilution potential E_d (calculated) (E_2-E_1)
			mv	mv	mv
M.Z.,	Healthy		41.3		
♂, 24			41.4	39.2	
				39.0	
			41.6		
			41.2		
		Mean	41.4	39.1	−2.3
After hyaluronidase (30 min)					
			51.3		
			51.3	44.4	
				44.0	
			52.5		
			52.6		
		Mean	51.9	44.2	− 7.7
O.T.,	Untreated				
♀, 41	scleroderma		35.1		
			35.3	25.8	
				26.1	
			35.9		
			35.5		
		Mean	35.5	26.0	− 9.5
After hyaluronidase (20 min)					
			44.2		
			44.0		
				31.5	
				31.9	
			43.0		
			43.8		
		Mean	43.8	31.7	−12.1

* Sign of potentials E_1 and E_2 was always positive, i.e., dermal connective tissue positive with respect to finger epidermis. Dilution potentials were calculated from the average values of E_1 and E_2. Each reading of E_1 and E_2 refers to a different isotonic or dilute solution.

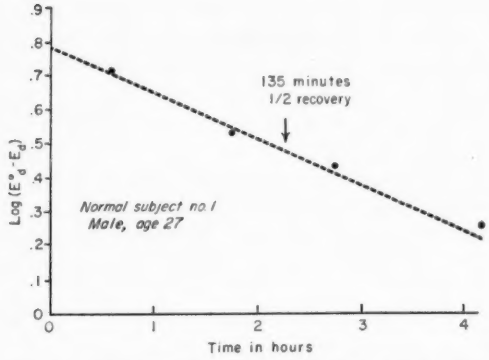

FIG. 2. LOGARITHMIC RECOVERY CURVE FOR A NORMAL SUBJECT.

to a value of 0.035 Eq, or to a dilution potential of − 5 mv. In the control series, potentials were observed over a period of 3 to 4 hours after the minimal potentials had been observed. In every case half-recovery had occurred within that time. The average half-recovery period for 10 normal subjects was found to be 156 minutes (Table I).

On the basis of animal experiments it has been found that recovery of colloidal charge after the action of hyaluronidase follows a logarithmic equation (16). It follows that half-recovery times may be conveniently estimated from a small number of serial measurements of dilution potential (Figure 2). It is assumed that at any time t after the removal of the enzyme, the rate of recovery is proportional to the displacement of x from the initial value, x^0. This is represented by the formula (15, 16):

$$dx/dt = a\ (x^0 - x) \qquad [6]$$

where a is a rate constant expressed in reciprocal minutes. By integration

$$\ln\ (x^0 - x) = -at + C \qquad [7]$$

where C is the integration constant, and ln is the natural logarithm. By applying Equation 5, the equation for E_d as a function of time is obtained:

$$\ln\ (E_d^0 - E_d) = -at + B \qquad [8]\ ^2$$

where the constant B is obtained from the maximum displacement $(E_d^0 - E_d)$ at zero time (beginning of recovery). In the computations it is convenient to use common rather than natural logarithms. Then

$$\log_{10}\ (E_d^0 - E_d) = -kt + \log_{10}\ (E_d^0 - E_d') \qquad [9]$$

where E_d is the dilution potential at time t, E_d^0 is the value before use of hyaluronidase, E_d' is the value of the maximally displaced dilution potential at zero time, and k is a rate constant expressed in reciprocal minutes. The relation between k and a is: $a = 2.303\ k$, where 2.303 is the conversion factor between common and natural logarithms. Equation 9 has been found to give an adequate description of the data in a large number of animal experiments (15, 16). Half-recovery time and the rate constant, k, are related by the formula: $t_{\frac{1}{2}} = 0.301/k$, where 0.301 is the logarithm of 2, and $t_{\frac{1}{2}}$ is the half-recovery time. From the data for 10 healthy subjects, values of k and $t_{\frac{1}{2}}$ were calculated. This requires values of E_d^0, E_d' and of the serial dilution potentials E_d as a function of time after maximal enzyme effect. The mean half-recovery time (Table I) agrees well with that estimated from the normal time-response curve shown in Figure 2.

Connective tissue diseases. In patients with connective tissue diseases, the negative charge density, x, of the dermal colloids was about 30 to 60 per cent lower than that found in healthy subjects. The mean value was approximately 35 mEq in

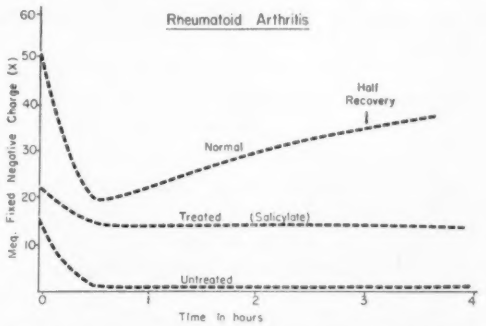

FIG. 3. MEAN TIME-RESPONSE CURVE FOR HEALTHY SUBJECTS COMPARED WITH CURVES OBSERVED IN A PATIENT WITH RHEUMATOID ARTHRITIS BEFORE AND AFTER TREATMENT. (Curves labeled, respectively, "normal," "untreated," and "treated.") Hyaluronidase was injected after the measurement at zero time.

[2] Equation 8 is used empirically to give a convenient means of estimating half-recovery times. A more exact check of the validity of the equation would require experimental data extending over more than one logarithmic decade. Since the potentials all fall between − 2 and − 12 mv, it is not possible to check the formula beyond these limits.

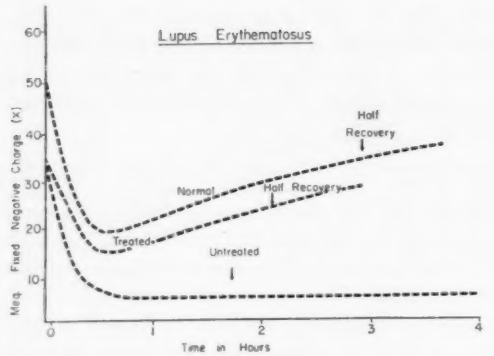

FIG. 4. TIME-RESPONSE CURVES FOR A PATIENT WITH LUPUS ERYTHEMATOSUS BEFORE AND AFTER TREATMENT WITH 50 MG OF PREDNISONE DAILY FOR 10 WEEKS.

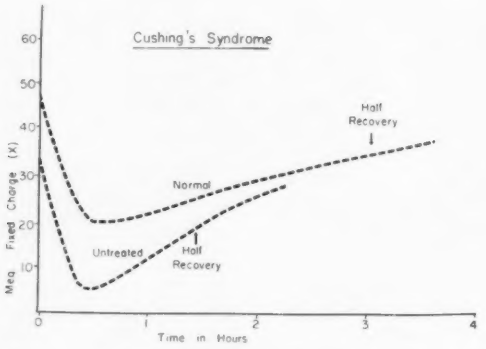

FIG. 6. TIME-RESPONSE CURVE FOR A PATIENT WITH CUSHING'S SYNDROME COMPARED WITH THE CURVE FOR NORMAL SUBJECTS.

patients with systemic lupus erythematosus, and about 15 mEq in patients with scleroderma and rheumatoid arthritis, as compared with the normal value of 50 mEq. After hyaluronidase injection, the value of x fell to about 5 mEq or less in all patients (Figures 3–6; Table I). This effect was observed in about 30 minutes. Usually no recovery was observed in any of the patients during study periods of up to 3.5 hours.

Cushing's syndrome. The connective tissue function test was also performed in three patients with Cushing's syndrome. The initial colloidal charge density was about 30 mEq, as compared with the normal level of 50. After hyaluronidase the level fell to about 5 mEq (Figure 6; Table I). Recovery was very rapid, however, and the initial negative charge was fully restored within 2 to 3 hours, while the half-recovery time was about 60 minutes.

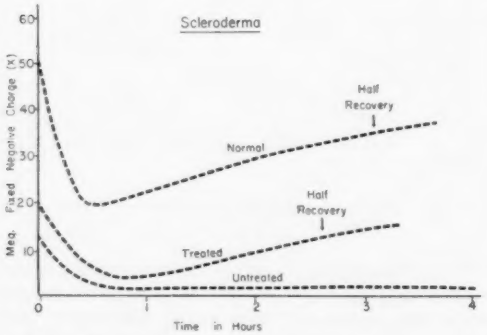

FIG. 5. TIME-RESPONSE CURVES FOR A PATIENT WITH SCLERODERMA BEFORE AND AFTER TREATMENT WITH 20 MG PREDNISONE DAILY FOR 6 MONTHS.

Effects of treatment. One patient with rheumatoid arthritis was restudied after 2 weeks of treatment with sodium salicylate (600 mg four times daily). The time-response curve is compared with that of the untreated group in Figure 3. Although the initial density of negative charge was slightly higher after therapy, the level was still considerably below the normal control level. Hyaluronidase produced only a slight change in this level, presumably due to the inhibitory effect of salicylate (19). No recovery was noted during the 3.5-hour observation period. The patient continued to receive salicylate for another week and was then retested 36 hours after discontinuation of treatment. The results at that time closely approximated the original curve observed previous to any treatment.

One patient with systemic lupus erythematosus was given prednisone (50 mg daily) for 10 weeks before retesting. After this treatment the initial negative charge density was not significantly changed. The change after hyaluronidase remained greater than normal, but was less than in the untreated patients. The recovery curve paralleled the normal, but remained approximately 5 mEq lower throughout (Figure 4).

Three patients with scleroderma were restudied after 3 to 12 months of treatment with prednisone (20 to 25 mg daily). The initial density of negative charge had increased by about 10 mEq. The change after hyaluronidase was slightly less than in the untreated cases, and some recovery was observed after the initial effect of the enzyme (Figure 5).

One patient with Cushing's syndrome was re-examined approximately 8 months after treatment by bilateral adrenalectomy. At that time he was receiving 37.5 mg of cortisone daily, and 25 mg of deoxycorticosterone acetate per month, and was in good health. The initial density of negative charge of the dermal connective tissue was about 14 mEq less than before surgery, and no recovery was observed during a 3.5-hour testing period after the action of hyaluronidase.

DISCUSSION

Attempts have been made from time to time to evaluate connective tissue changes in pathological states by investigating the spreading of various diffusible substances. One of the earliest methods, proposed by McClure and Aldrich (*20, 21*), measured the disappearance time of intradermal saline blebs. The results may be interpreted as reflecting indirectly the state of the tissue colloids, including their water-binding properties.

More recently several procedures using hyaluronidase and dyes have been described (*22–25*). The spreading reaction appears to be very complex; it depends not only on the state of the tissue but also on the method of injection, the affinity of the tissue for the indicator, and the degree of irritation produced by the dye (13). Moreover the effect of recovery on the spreading reaction is difficult to delineate. For these reasons the interpretation of dye diffusion experiments may be equivocal. The colloidal state of the ground substance can be more directly studied by using the electrometric method outlined in this report. Kinetics of the response can be quantitatively followed. In this way the stages of breakdown and recovery are sharply defined.

In untreated patients a clear distinction between the various connective tissue diseases cannot be made on the basis of electrometric measurements. When the time-response curves are compared with the normal curve, however (Figures 3–5), three characteristics of these pathological conditions may be distinguished: *1*) the initial colloidal charge density is lower in the patients with rheumatoid arthritis, scleroderma and systemic lupus erythematosus; *2*) the colloidal charge density after hyaluronidase becomes very low (5 mEq or less) compared with that found in the healthy group under similar conditions (about 20 mEq); and *3*) there is little or no reversal of the effect during the recovery period of 3 to 4 hours.[3] These findings suggest that in these disease states the ground substance of the dermal connective tissue differs from the normal both in composition and in responses. Changes in the hexosamine-hydroxyproline ratios of dermal connective tissue in certain diseases support this interpretation (*26*). Reversal of the enzymatic breakdown appears to depend on both cellular and extracellular responses; synthesis of extracellular colloids would depend on cellular metabolism, whereas replacement by flow or hydrostatic pressure would depend primarily on the viscous properties of extracellular phases and components. In connective tissue diseases there appears to be a decrease in negatively charged colloidal components of the kind which are broken down by hyaluronidase (i.e., chondroitin sulfate and hyaluronic acid). Accompanying this change there is a marked decrease in the rate at which negatively charged components are replaced in a region in which they have been enzymatically hydrolyzed. The inhibited rate may result from impaired rheological properties as well as from failure of the cells to synthesize the necessary components. The half-recovery time as determined electrometrically is much more rapid than results obtained by isotopic studies of hyaluronic acid (4 days) and chondroitin sulfuric acid (8 to 10 days) (*27*). Clinical and histological observations on wound repair, however, also indicate a more rapid recovery rate. In the electrometric studies the recovery is influenced by the flow of colloids from contiguous areas and by cellular synthesis of new materials. Fluidity seems to be enhanced in Cushing's syndrome or by administration of cortisone (14), and to be lowered in collagen diseases.

The results of therapy, particularly when the same drug was used, show that there are also characteristic differences between the various conditions. The time-response curve in the patient with systemic lupus erythematosus receiving prednisone approximated the normal curve fairly well, in contrast with the time-response curves of patients with scleroderma who were receiving similar treatment. This difference in response is in accordance with

[3] The possibility that changes in connective tissue state would be demonstrated in other kinds of illness is recognized but has not been tested.

clinical experience. Electrometric changes do not, however, always parallel the clinical response to therapy. In the case of rheumatoid arthritis, for example, salicylates produced remission of symptoms with relatively small changes observed in the state of the tissue colloids, as measured by colloidal charge. It is evident that "clinically successful" treatment is not always synonymous with reversal of the pathological tissue changes.

Whereas under normal circumstances a compound such as cortisone retards repair, it promotes a remission of symptoms in many connective tissue diseases. The following explanation is suggested. Cortisone stimulates the connective tissue cells to form ground substance which differs qualitatively from the normal and which is characterized by a low density of negative colloidal charge (low cation binding). This has been demonstrated in electrometric studies on animals (14) and in the patients with Cushing's syndrome. In the normal subject the altered ground substance formed in response to cortisone interferes with proper healing. In the pathological state, however, the same modified ground substance represents a partial reversal of the disease process. In the healthy subject the modified ground substance is characterized by lowered negative charge and by increased fluidity; in pathological states cortisone may increase both the negative charge and the fluidity. Thus, depending on the initial state of reference, cortisone may promote either a partial degradation or a partial restoration of the connective tissue.

The adaptation of the electrometric method as a test of connective tissue function in patients has made it possible to characterize the state of the colloids, to describe some of the recuperative powers of the tissue, and to determine the response to drugs. Through *in vivo* studies of the physicochemical properties of connective tissue, valuable insights into its normal and pathological behavior may be gained.

SUMMARY

The rate at which connective tissue ground substance is disaggregated and restored after injection of hyaluronidase may be taken as an index of its initial state and its capacity for repair. This reaction was studied by an electrometric method involving the serial measurement of displaced Donnan potentials in the dermis. Normal persons and patients with rheumatoid arthritis, scleroderma and systemic lupus erythematosus were compared. The results indicate that in these connective tissue diseases a ground substance is formed which differs both qualitatively and quantitatively from the normal. The rate of reconstitution after enzymatic action is markedly delayed. The administration of corticosteroids tends to reverse these conditions. The measurements of changes in the state of the ground substance after the injection of hyaluronidase is a useful test for evaluating connective tissue diseases and the effects of therapy.

ACKNOWLEDGMENT

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ACCELERATED SKIN GRAFT REJECTION IN HUMANS PRE-IMMUNIZED WITH HOMOLOGOUS PERIPHERAL LEUKOCYTES *

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Delayed intradermal sensitivity to homologous white blood cells has been described in humans rejecting full thickness skin homografts (1). The reaction at the site of the challenge leukocyte was greatest at 18 to 24 hours against the cell suspensions obtained from the skin donor. It was noted, however, that lesser reactions were consistently elicited to similarly prepared and simultaneously injected leukocytes obtained from donors other than the sensitizing skin donor. There was no recipient response to injections of autologous leukocytes, homologous erythrocytes or homologous plasma. Biopsy sections taken at peak reaction times were similar to early tuberculin responses. It was postulated that the skin erythema and induration were a manifestation of an antigen-antibody reaction, in which antibodies developed against the skin homograft were capable of also reacting with shared antigen(s) within or upon the peripheral white blood cell. To explain the lack of specificity of the skin response, it was assumed that there is a distribution of common transplantation antigens in the human, as has been shown by Berrian and Jacobs in the mouse (2). The present experiment supports this supposition and suggests that transplantation immunity in the human is not as specific a phenomenon as has been previously proposed (3).

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‡ Fellow of the National Foundation.

§ Investigator, Howard Hughes Medical Institute.

MATERIALS AND METHODS

Eighteen ambulant adult volunteers received full thickness skin homografts on the medial surface of the upper arm, after inoculation with homologous leukocytes. All of the subjects had negative past histories for malaria, jaundice and syphilis. In addition to the physical examination, they were subjected to the usual screening procedures required for donors to a hospital blood bank. The subjects had never received blood transfusions and were free of diseases known to alter immunologic reactivity. ABO and CDE blood groups were determined prior to immunization (Table I). Donor leukocytes were harvested from freshly drawn peripheral venous blood by a dextran sedimentation technique (4). The leukocytes employed were harvested prior to administration. The time lag before injection was related to preparation and varied from slightly under 1 hour to a maximum of 3 hours. In each instance the white blood cells were resuspended in donor plasma, in a total volume of 3 ml. The final sediment was counted and measured and an aliquot was chosen so that 3 ml (30 injections) contained the desired dose. Duplicate chamber counts of the leukocyte yield enabled adjustment of the volume of inoculum so that the predetermined number of white blood cells could be administered. The total number of leukocytes injected varied from 2×10^7 to 48×10^7 (Table II). Subjects 1 and 2 received four subcutaneous doses of 12×10^7 leukocytes at 7-day intervals for a total dose of 48×10^7 leukocytes. On Day 29 of the experiment, 1 day after the last injection, these subjects were grafted with skin from the leukocyte donor and from an unrelated person. As a control of surgical technique, an autograft was placed in close proximity to the two homografts. Because subcutaneous immunization failed to induce accelerated graft rejection, the experimental protocol was changed for Subjects 3 through 16. These volunteers received, as their immunizing dose, 30 intracutaneous leukocyte injections on the back and thighs. Each injection contained, in a volume of 0.1 ml, one-thirtieth of the total leukocyte dose which varied from 2×10^7 to 30×10^7 (Table II). Six days after the multiple intracutaneous inoculations, donor and nonspecific skin grafts were placed as in Subjects 1 and 2 (Figures 1 and 2).

TABLE I

Race, sex and donor blood types in 18 patients immunized with leukocytes

Patient no.	Recipient			Leukocyte donor			Nonspecific donor		
	Race	Sex	Blood type	Race	Sex	Type	Race	Sex	Type
1	N	F	A+	N	M	B+	N	F	A+
2	N	M	B+	N	F	A+	N	F	A+
3	W	F	O+	W	M	B+	W	F	B+
4	W	M	B+	W	F	O+	W	F	B+
5	W	M	A+	W	M	O+	Not done		
6	W	M	O+	W	M	A+	Not done		
7	W	M	A+	W	M	B−	W	M	O+
8	W	M	B−	W	M	A+	W	M	O+
9	W	M	O+	W	M	O+	W	M	O+
10	W	M	O+	W	M	O+	W	M	O+
11	W	M	A+	W	M	A−	W	M	O+
12	W	M	A−	W	M	A+	W	M	O+
13	W	M	A+	W	M	A−	W	M	O+
14	W	M	A−	W	M	A+	W	M	O+
15	W	M	A+	W	M	O+	W	M	O+
16	W	M	O+	W	M	A+	W	M	O+
17	W	M	B−	W	M	A+	W	M	O+
18	W	M	A+	W	M	B−	W	M	O+

All grafts were full thickness skin removed under local anesthesia from the medial aspect of the upper arm and sutured into the host bed with interrupted 5-0 nylon sutures. The wounds were covered with a nonadherent plastic dressing which facilitated frequent inspection, photography and biopsy. In the first 6 patients studied, all of the grafts were 2.5 cm². From the data obtained in this group, and from another concurrently studied series (5), it was believed that sufficient information could be gained from a smaller graft to warrant a reduction in size to 1.0 cm². The excellent autograft survival in the earlier volunteers caused us to discontinue simultaneous control autografts in Subjects 7 through 18. Material was obtained by biopsy of the graft only and not of the graft bed. In all cases two biopsies were taken at Days 6 or 7 and another on Day 9 or 10. This allowed a mi-

TABLE II

Type of rejection in 18 patients preimmunized with homologous leukocytes

Subject	No. of leukocytes administered $\times 10^7$	Route of injection	Rejection pattern	
			Leukocyte donor graft	Nonspecific*
1	48.0	Subcutaneous	First set	First set
2	48.0	Subcutaneous	First set	First set
3	10.0	Intracutaneous	White graft	Accelerated
4	7.2	Intracutaneous	Accelerated	First set
5	6.5	Intracutaneous	Accelerated	Not done
6	9.0	Intracutaneous	White graft	Not done
7	20.0	Intracutaneous	Accelerated	First set
8	20.0	Intracutaneous	White graft	Accelerated
9	20.0	Intracutaneous	White graft	White graft
10	20.0	Intracutaneous	Accelerated	First set
11	12.0	Intracutaneous	Accelerated	First set
12	12.0	Intracutaneous	White graft	White graft
13	2.0	Intracutaneous	Accelerated	Accelerated
14	2.0	Intracutaneous	First set	First set
15	30.0	Intracutaneous	White graft	White graft
16	30.0	Intracutaneous	Accelerated	Accelerated
17	12.0	Intracutaneous	White graft	Accelerated
18	12.0	Intracutaneous	White graft	Accelerated

* In two instances, the nonspecific graft was an unusually prolonged first set rejection. In Subjects 11 and 14 active rejection was observed to commence by Day 21 after grafting.

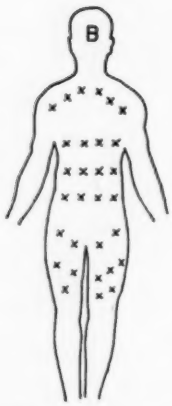

FIG. 1. APPROXIMATE LOCATION OF THE MULTIPLE INTRADERMAL LEUKOCYTE INJECTIONS EMPLOYED TO IMMUNIZE SUBJECTS 3 TO 18. Subject B was intradermally inoculated with leukocytes of Subject A at 30 sites (total volume of inoculum is 3 ml, containing 2 to 30×10^7 leukocytes).

croscopic assessment of the type of rejection; in two cases showing slow rejection, biopsies also were taken on Days 17 and 19, respectively.

Grading of the rejection process

In order to assess properly the changes in altered skin grafts, a series of unmodified first set, accelerated, and white graft rejections was carefully studied (5), by daily inspection and biopsy. A brief summary of the criteria

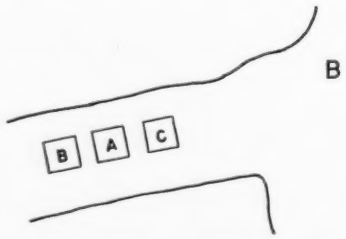

FIG. 2. LOCATION OF DONOR AND NONSPECIFIC SKIN GRAFTS. Six days after multiple intradermal leukocyte injections, Subject B was challenged with full thickness skin grafts from Subjects A and C. B, a control autograft, was also placed. Only the first 6 subjects were given autograft controls. The nonspecific homograft was omitted in Subjects 5 and 6.

used to determine the type of rejection encountered may be subdivided into two sections.

Gross findings. Typical of the first set rejection is early (2 to 3 days) vascularization, detectable by a pink coloration, blanching when pressure is applied, and brisk bleeding upon incisional biopsy. Thereafter, the first set rejection and the autograft are indistinguishable until the sixth to seventh day when the homograft assumes a cyanotic color, fails to blanch on pressure, and has obvious interstitial edema. During the next 5 to 9 days, the now avascular homograft gradually changes into a crusty black scab (Figures 3 and 4).

A white graft, by contrast, never establishes graft-bed vascular anastomosis, the total lack of blood supply being responsible for its peculiar pallor. In time, the white

FIG. 3. PHOTOGRAPH OF AUTOGRAFT (LEFT), LEUKOCYTE DONOR SKIN (CENTER) AND NONSPECIFIC SKIN (RIGHT), ON THE FIFTH POSTGRAFTING DAY IN SUBJECT 2. The photograph demonstrates no discernible difference between the auto- and homografts. In this case, subcutaneous leukocyte injections failed to induce homograft immunity.

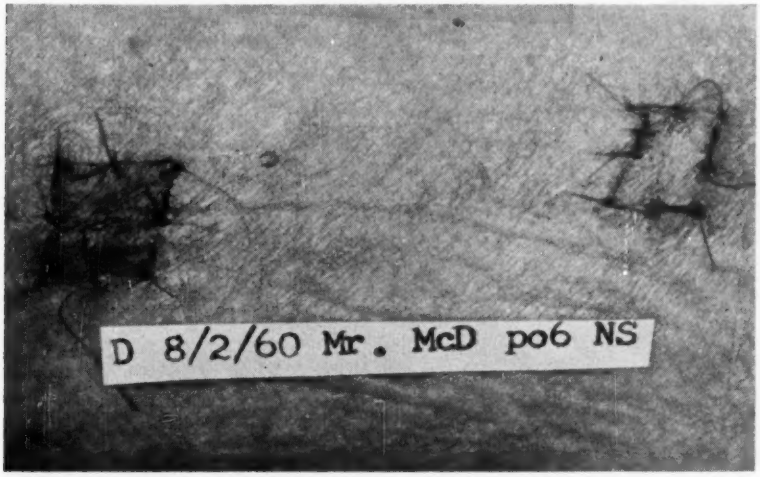

FIG. 4. SUBJECT 10. The leukocyte donor graft (left) is edematous and cyanotic by the sixth postgrafting day, indicating accelerated rejection. The nonspecific graft (right), placed simultaneously 6 days after intradermal leukocyte immunization, resembles the surrounding host skin and is undergoing a first set rejection.

graft appears to melt away as a result of a sterile proteinolysis, although its color remains pale gray as new epithelium granulates in from the surrounding bed margins. Both its striking color and its failure to bleed when incised easily identify the white graft (Figure 5).

The accelerated or second set rejection pattern rapidly recapitulates the events of a primary rejection. After transient but clear early vascularization, cyanosis and edema are discerned by the fourth postgrafting day. By the eighth day the florid rejection is completed and a hard scab remains (Figures 4 and 6).

Microscopic correlation. No distinction between autograft and first set homograft biopsy material is possible for the first 6 days. Further sections of autografts progressively display a gradual return to the appearance of normal skin. Increasing numbers of perivascular lympho-

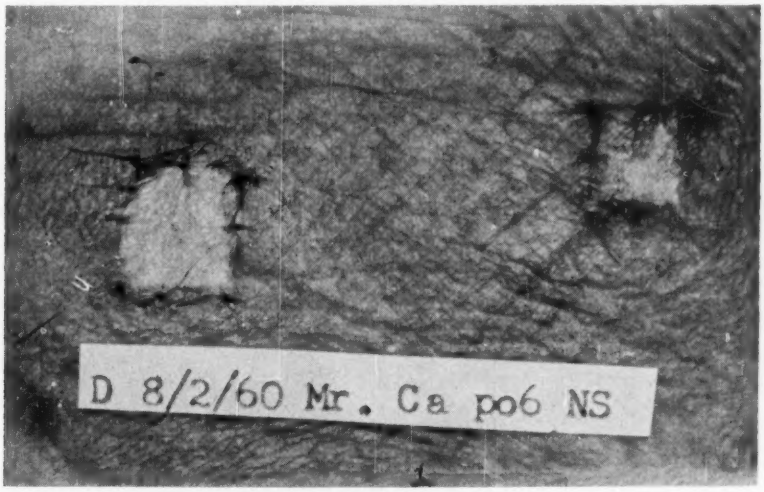

FIG. 5. SUBJECT 8. This subject evinced white graft rejections of leukocyte donor skin (left), and nonspecific skin (right), after intracutaneous preinjection with homologous leukocytes 6 days before grafting. The white graft is interpreted as a manifestation of a very intense transplantation immunity.

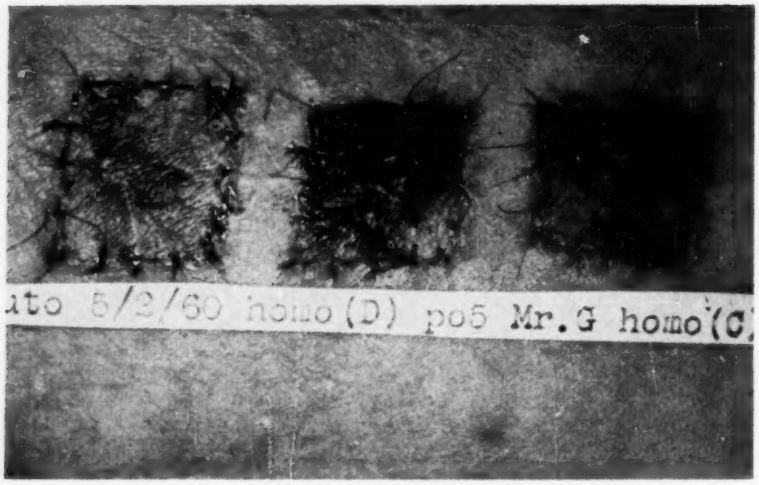

FIG. 6. SUBJECT 4. After the intracutaneous inoculation of 7.2×10^7 leukocytes 6 days prior to grafting, Subject 4 rejected both the leukocyte donor skin (center) and nonspecific control graft (right) in an accelerated fashion. Cyanosis and interstitial edema are present in both homografts while the autograft (left) appears healthy, with a well healed biopsy site in the upper left-hand corner. By Day 5, easy distinction can be made between accelerated and first set rejections (see Figure 3).

cytes, gradually extending into the dermis and epidermis, represent the primary rejection on the seventh to eighth day (Figures 7 and 8). Extensive vascular thrombosis supervenes on the eleventh or twelfth day. Symbolic of a white graft type of rejection is an early polymorphonuclear cell permeation of the dermis and epidermis of the totally avascular graft (Figure 9). The accelerated or second set rejection has a blood supply for only 2 to 3 days. The skin biopsies of grafts undergoing accelerated rejection showed no vascular thrombosis. Presumably the vessels at the junction of graft host tissues are primarily involved, but in this study this junctional area was not examined, since it would have required local anesthetic for each biopsy procedure. There is only slight cellular infiltration prior to the death of the graft on the seventh or eighth day (Figure 8). Patterns intermediate

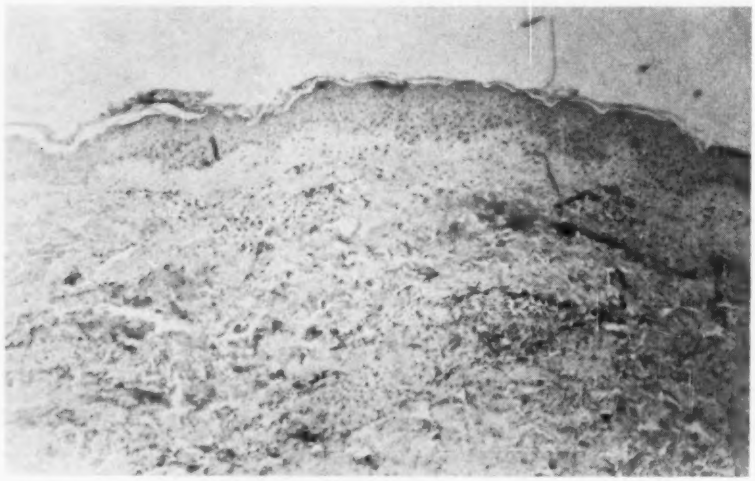

FIG. 7. SUBJECT 7. The earliest sign of primary or first set rejection is perivascular lymphocytic infiltration, here observed on the sixth postoperative day in the nonspecific graft placed on Subject 7 (H&E, × 55).

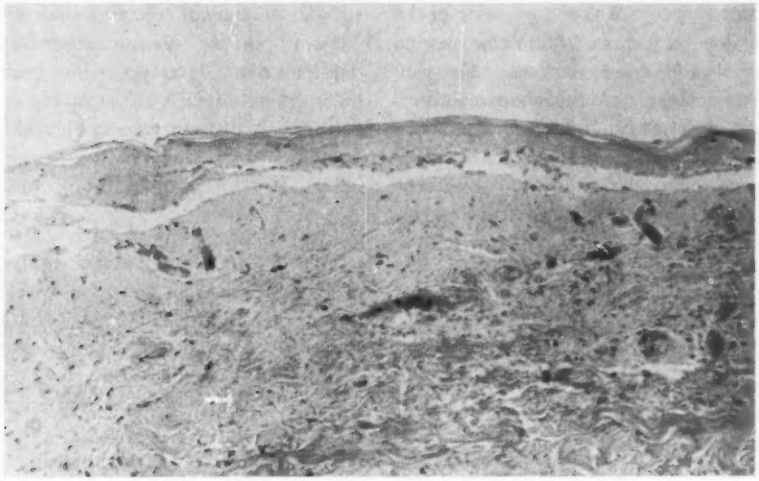

FIG. 8. SUBJECT 4. Contrast the first set rejection shown in Figure 7 with the features of accelerated rejection noted in this biopsy of the nonspecific graft placed on Subject 4 also taken on the sixth postgrafting day. A dead epidermis, dilated blood vessels and the absence of cellular infiltration connote an accelerated rejection (H&E, × 55).

between accelerated and white graft have been observed twice. In these grafts, the gross appearance determined the category ascribed.

RESULTS

There were no technical failures in any of the grafts in this series. Technical failure of a graft is usually due to an underlying hematoma. The graft is raised from the bed and the clot is discovered on biopsy. The graft undergoes escharification and does not have the appearance of a "white graft," which always remains flat. On microscopic section the polymorphonuclear infil-

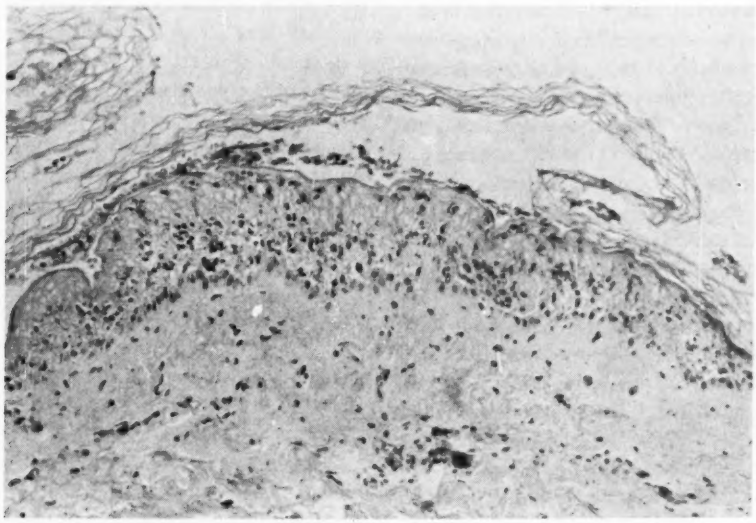

FIG. 9. SUBJECT 3 ON SIXTH POSTGRAFTING DAY; SPECIFIC GRAFT. Diagnostic of a white graft rejection is the early death of the epidermis coupled with dense diffuse infiltration with polymorphonuclear leukocytes (H&E, × 110).

trate, typical of a white graft does not occur in the graft which has been a technical failure because of separation from its bed by a hematoma. The subjects withstood the stresses of multiple inoculations and subsequent skin grafting without untoward event. None of the volunteers suffered temperature elevation, regional adenopathy or wound infection. The third and fourth subcutaneous injections in Subjects 1 and 2 caused the formation of hot sterile abscesses which proved painful for several days although surgical drainage was not necessary. An uncomfortable, local stinging sensation, lasting about 5 minutes, usually followed the intracutaneous inoculations.

Subcutaneous immunization. Repeated subcutaneous injections of a total of 48×10^7 leukocytes prior to grafting with leukocyte donor and nonspecific skin failed to elicit accelerated rejections of any of the homografts placed on Subjects 1 and 2. The gross signs of a first set rejection were apparent in the leukocyte donor skin on Subject 1 on Day 10, and in the nonspecific skin on Day 8. Similarly, Subject 2 evinced a primary rejection of leukocyte specific skin on Day 9, and of nonspecific skin on Day 8. In both instances, the autografts survived normally.

Intracutaneous immunization. Of the 15 patients pretreated with intradermal homologous leukocytes, all but 1 had altered immunologic rejections of subsequent full thickness skin homografts. Illustrative of the modified responses were the white grafts observed in 8 of the 16 leukocyte donor skin grafts in this group. Accelerated second set rejections were noted in 7 of the leukocyte donor skin grafts (Table II). One patient experienced an unmodified first set rejection of the skin graft from the donor of the pregrafting leukocytes. In 14 instances an additional homograft was placed from a donor whose leukocytes had not been used to presensitize the recipient. This nonspecific skin graft was placed at the same time as the graft from the donor whose leukocytes had been injected 6 days previously. Of these 14, white grafts occurred 4 times, accelerated rejections 6 times, and primary or first set rejections were noted in 4 subjects. Examples of each of the rejection patterns encountered are included in Figures 3–9.

There was no obvious correlation between the ABO and CDE blood groups of donor and recipient and the type of rejection response encountered. Thus, O positive patients immunized with O positive leukocytes developed white graft rejections as did recipients who had major blood group incompatibility with their respective leukocyte and skin donors.

DISCUSSION

The pioneer studies of Medawar incontrovertibly established the immunologic basis of homograft rejection (6). It is now known that the ability to reject a second homograft in an accelerated fashion is a fundamental biological process found in all species studied. Early experiments suggested that the homograft reaction is an individual specific response (6). Medawar was able to immunize an animal against skin homografts by the prior injection of blood leukocytes (7), and Billingham, Brent and Medawar showed that second set rejections followed preimmunization of the host with liver, spleen or kidney cell suspensions (8). These same workers later demonstrated that cells totally disintegrated by ultrasonication retained their power to elicit skin transplantation immunity in mice (9).

Our preliminary efforts to locate human transplantation antigens within the circulating leukocyte were not successful. In several unreported experiments, volunteers repeatedly injected intramuscularly with donor whole blood evinced unmodified first set skin rejections. Likewise, Subjects 1 and 2 in this experiment had no gross or microscopic evidence of rejection acceleration subsequent to subcutaneous leukocyte pretreatment. The efficacy of intradermal immunization seen in our data offers further evidence of the variation of species response to the route of antigen administration. Thus, Billingham, Brent and Mitchison found all routes of administration of homologous cells tested in mice and guinea pigs immunologically equivalent in the induction of transplantation immunity, while the rabbit showed a markedly accentuated response to intradermal inoculation as compared with intravenous, subcutaneous or intraperitoneal routes (10).

A second explanation may in part account for failure of leukocyte administration by the subcutaneous route to sensitize the recipient.

The intradermally immunized patients exhibited considerably diversified rejection patterns. At

least two factors determine the intensity of homograft rejection: donor-recipient genetic dissimilarity and the immunizing dosage. Berrian and Jacobs were able to predict the timing of third strain skin homograft rejection in purebred mice following crossed immunization of two other pure strains with splenic cell suspensions (2). By repeated interstrain grafting they were able to calculate the number of transplantation antigens shared by donor and recipient and correlate the onset and intensity of rejection with the degree of genetic disparity of the engrafted pairs. Little is known concerning the number or distribution of human transplantation antigens (11). Merrill and co-workers reported the prolonged survival of a full thickness skin homograft placed on the upper arm of a fraternal twin who shared 23 blood groups with his brother-donor (12). Blood group antigens and transplantation antigens are not, however, inherited *pari passu*. Woodruff and Allan (13) have reported a normal first set rejection between two unrelated individuals with 10 identical blood group antigens. We have seen a normal first set rejection in skin transplanted from one nonidentical twin to another when all but 1 of 27 blood group antigens were identical. It is reasonable to assume that the different types of rejection encountered in several of our patients who received the same number of donor leukocytes was due to the heterogeneity of transplantation antigen distribution in man. In this light, the significance of the unusual prolongation of two nonspecific first set reactions (Table II) is unclear. A recent report by Rapaport, Thomas, Converse and Lawrence describes the prolongation of skin graft survivals in humans "typed" for similarity of transplantation antigens (14). The similarity of transplantation antigens in this instance was surmised when a skin graft from one member of the donor-recipient pair caused accelerated rejection of skin from the second member when an indifferent recipient was used for both grafts. The fact that skin from one accelerated the rejection of skin from another and that subsequently skin exchanged between the two, showed an unusually long survival, which was presumed to be due to a sharing of similar transplantation antigens. A similar observation was made by Kuss and LeGrain (15) in unrelated individuals between whom a kidney transplant with prolonged survival was subsequently done.

There is limited information concerning gradation of rejection intensity dependent upon increasing antigen dosage. Zotikov, Budik and Puza described shorter rejection times with increased size of rat homografts (16). They noted that as the size of the homograft was increased to greater than one-third of the recipient's total skin surface area, "immunoparalysis" with greatly prolonged graft survival was induced. No data pertinent to dose response are available for man. Our series is too small to allow statistical analysis although the data suggest that the degree of immunity evoked by intracutaneous leukocytes injections is, at least in part, dose related. None of the 4 subjects inoculated with less than 9×10^7 leukocytes responded with white grafts to either leukocyte donor or nonspecific challenge grafts, while 8 of 12 patients given at least 9×10^7 leukocytes developed white graft reactions in the graft from the leukocyte donor. Rapaport and associates believe the white graft to be "either a qualitatively different or a more intense form of immune response against foreign tissue perhaps mediated by serum antibody" (3). The importance of circulating antibody in homograft rejection is unclear (17, 18), and its contribution to the formation of the white graft is as yet unknown. If a dose-related antigenic response is present in man, as we believe, then this experiment lends credence to the concept that the white graft is an expression of a greater level of immunity than is an accelerated rejection.

To support this hypothesis further, it is noted that the intensity of rejection in the nonspecific grafts was in each case equal to or less than the degree of immunity displayed in the specific leukocyte donor skin. A white graft in the leukocyte donor graft was accompanied by either a white graft or an accelerated rejection in the nonspecific skin. Analogously, a specific accelerated rejection complemented either an accelerated or first set response in the nonspecific graft.

Therefore, the weaker reactions to nonspecific grafts would be in accord with partial sharing of transplantation antigens between leukocyte donor and nonspecific skin samples. This would also concur with Rapaport and co-workers' study of white graft production in which five of eight non-

specific grafts exhibited significantly reduced survival time (3).

The difficulties inherent in investigations of human transplantation immunity may be somewhat eased by additional attention to the circulating leukocyte. Lawrence, Rapaport, Converse and Tillett meticulously detected the presence of a "factor" capable of transferring homograft immunity in extracts of peripheral white blood cells (19). The data presented here indicate the presence within the same cell population of antigenic constituents competent to induce the known forms of skin homograft rejection.

SUMMARY AND CONCLUSIONS

The intradermal inoculation of peripheral leukocytes prior to full thickness skin grafting induced white graft and accelerated rejection of first set homografts in the human. Simultaneously placed skin grafts obtained from patients other than the leukocyte donor were also rejected in an accelerated fashion, indicating that transplantation immunity in the human is not individual specific. It is postulated that the type of skin graft rejection encountered in this experiment was determined by both the dose of immunizing leukocytes administered and the genetic similarity between donor of immunizing leukocytes and donor of the nonspecific skin graft.

ACKNOWLEDGMENT

We are indebted to Professor Peter B. Medawar for his valuable comments pertinent to the experimental design; Mr. David McMakin offered tireless technical assistance; Dr. Thomas A. Warthin and Dr. Richard A. Warren kindly made available the facilities of the West Roxbury Veterans Administration Hospital where Mr. M. C. Cheney prepared several of the illustrations. Miss Audrey J. Clemens, R.N. handled with warmth and dignity many of the innumerable problems inherent in human experimentation.

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THE ROLE OF PLASMA FREE FATTY ACIDS IN DEVELOPMENT OF FATTY LIVER

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The accumulation of fat in the liver under many diverse experimental conditions has frequently been used as an index of fat mobilization from adipose tissue (1–6). In recent years, it has become clear that the free fatty acids (FFA) of the plasma represent a major transport form for mobilization of lipid from adipose tissue (7, 8) and methods are now generally available for measurement of this important plasma lipid component (7, 9, 10). Therefore, it is now possible to examine more directly this inferred relationship between mobilization of fat and the development of fatty liver. The present experiments were designed to determine whether increasing the rate of mobilization of fatty acids from the periphery in normal animals might lead to development of fatty liver. Epinephrine causes an immediate and marked increase in the rate of release of FFA from adipose tissue *in vitro* (11) and *in vivo* (12), and norepinephrine acts similarly (13, 14). Because the FFA response to norepinephrine is smoother and more sustained than the response to epinephrine (13), the former has been used in most of these experiments.

METHODS AND EXPERIMENTAL PROCEDURE

Experiments were performed on male mongrel dogs weighing 8 to 15 kg, maintained for 1 to 3 weeks on a diet of canned meat [1] which was offered each morning for a period of 4 hours. Following a 24-hour fast, the animals, anesthetized with sodium pentothal (thiopental), received via the femoral vein an 8-hour infusion of norepinephrine [2] in saline (1 to 2 μg per kg per minute) while the control animals received only saline. The infusion was delivered by a Bowman pump at a constant rate of 1 ml per minute. Before starting the infusion, liver biopsies (1 to 2 g) were obtained through an abdominal incision which was closed before the infusion was begun.

[1] Red Heart Dog Food, John Morrel and Co.

[2] Parke, Davis and Co.

Portal vein infusions were administered by way of a small polyethylene catheter introduced into a splenic vein and threaded up to the portal vein at the time of laparotomy. At the end of the 8-hour infusion, a second liver biopsy was obtained through the same incision which was reopened for this purpose. In one experiment, serial liver biopsies were obtained at 0, 2, 4 and 8 hours during the course of a norepinephrine infusion. Serial blood samples were taken throughout each infusion. All blood samples were drawn from the femoral vein into dry syringes, transferred to heparin-containing tubes, promptly chilled, and kept at 4° C until analytical procedures were started. Plasma FFA were determined by the method of Dole (9). All biopsies were analyzed for total cholesterol (15), phospholipids (16), and total lipids (17), and examined histologically for fat using an Oil Red O stain. Triglycerides were calculated by the Bragdon method (17). The portions of the liver biopsies used for lipid analysis were homogenized in acetone-ethanol (1:1, vol/vol) and centrifuged. One aliquot of the supernatant was taken for cholesterol determination and the remainder was taken to dryness under nitrogen. The residue was taken up in chloroform-methanol (2:1), water was added to separate the two phases, and aliquots of the chloroform phase were taken for determination of phospholipids and total lipids. Blood glucose was measured colorimetrically by the Somogyi-Nelson method (18).

Liver lipids were fractionated into subclasses using a silicic acid column according to Borgstrom (19). The neutral lipids as a group were eluted with chloroform and the phospholipids with methanol. The lipids in the chloroform eluate were then applied to silicic acid-treated paper on which the cholesterol esters and glycerides were separated by using 99 per cent iso-octane, 1 per cent acetic anhydride as solvent (20). The lipid spots on the paper were identified by staining with a spray of 0.04 per cent Rhodamine B in methanol. Then the spots were cut out and eluted with chloroform-methanol (2:1), which was then evaporated to dryness in small glass tubes. One ml of methanol containing 2 per cent H_2SO_4 (vol/vol) was then added to each tube, the tubes were sealed, and the mixtures were heated at 65° C overnight. One ml of water was then added to each tube and the methyl esters were extracted into petroleum ether. The petroleum ether extracts were concentrated and aliquots were analyzed by gas-liquid chromatography. The column used was a U-shaped glass column, 4 mm I.D., 6

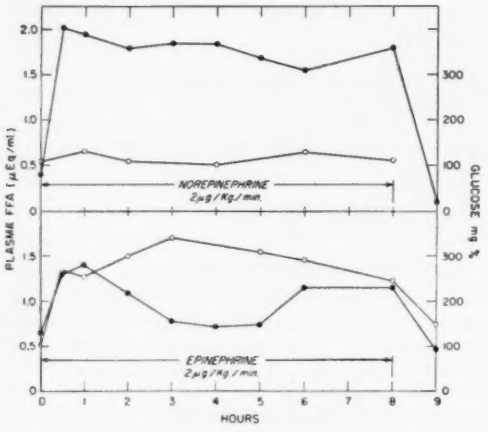

FIG. 1. PLASMA FFA (●——●) AND GLUCOSE (○——○) RESPONSES TO CONTINUOUS FEMORAL VEIN INFUSION OF NOREPINEPHRINE (ABOVE) AND EPINEPHRINE (BELOW). Same animal used for both studies.

feet long, packed with Chromosorb W (Johns Manville) on which a coating of ethylene glycol-adipate polyester, 10 per cent (wt/wt), had been applied. The analysis was performed at 190° C. The detector used was a modification of that described by Lovelock (21), the response of which had been calibrated for quantitative accuracy.

RESULTS

Comparative effects of epinephrine and norepinephrine on plasma FFA and glucose levels during an 8-hour infusion. Figure 1 represents the changes in plasma FFA and glucose levels during norepinephrine infusion (top half), and at a later date, and in the same dog, during epinephrine infusion (bottom half). The FFA response to norepinephrine was sustained at high levels throughout the 8-hour infusion, dropping precipitously when the infusion was terminated. The FFA response to epinephrine was prompt and marked but the maximal levels reached were not as high as those seen with norepinephrine. Despite continued infusion of epinephrine at the same rate, the FFA level fell to values not far above the control level but then rose again later in the experiment. These changes in FFA level appeared to bear a reciprocal relation to changes in blood glucose levels. Norepinephrine has insignificant glycogenolytic properties compared with epinephrine (22), and no rise in blood glucose is seen with the former. Norepinephrine was therefore chosen for the following experiments in which sustained FFA levels were desired.

Effect of an 8-hour infusion of norepinephrine on liver lipids. In Figure 2 the effect of an 8-hour infusion of norepinephrine on liver triglyceride concentration is illustrated. There was a progressive rise in liver triglyceride content throughout the infusion from an initial level of 4 to 5 mg per g liver to an 8-hour concentration of 33 mg per g. Associated with the increase in triglycerides was a marked elevation of the plasma concentration of FFA which continued until the infusion was terminated. The microscopic appearance of the liver is shown in Figure 3, in which a progressive rise in stainable fat is seen with a striking fatty infiltration by 8 hours.

Seven experiments were carried out in which the changes in plasma FFA concentration and the changes in liver lipid concentrations were measured over the course of an 8-hour infusion of norepinephrine. The FFA responses are shown in Figure 4 (E-K), along with the curves for FFA concentration in four control animals (A-D) receiving intravenous saline only. The corresponding changes in liver lipids are tabulated in Table I. All experimental animals (E-K) had a marked and sustained elevation of plasma FFA for the duration of the infusion, which was associated with increases in liver triglyceride as great as tenfold over the 8-hour period (mean increase, 773 per cent). The plasma FFA levels of the control animals (A-D) remained low and there was little or no change in liver triglyceride (mean increase, 39 per cent). The concentrations of liver cholesterol and phospholipids showed

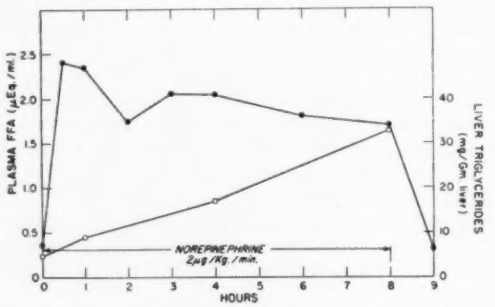

FIG. 2. PLASMA FFA (●——●) AND LIVER TRIGLYCERIDE (○——○) RESPONSES TO CONTINUOUS FEMORAL VEIN INFUSION OF NOREPINEPHRINE.

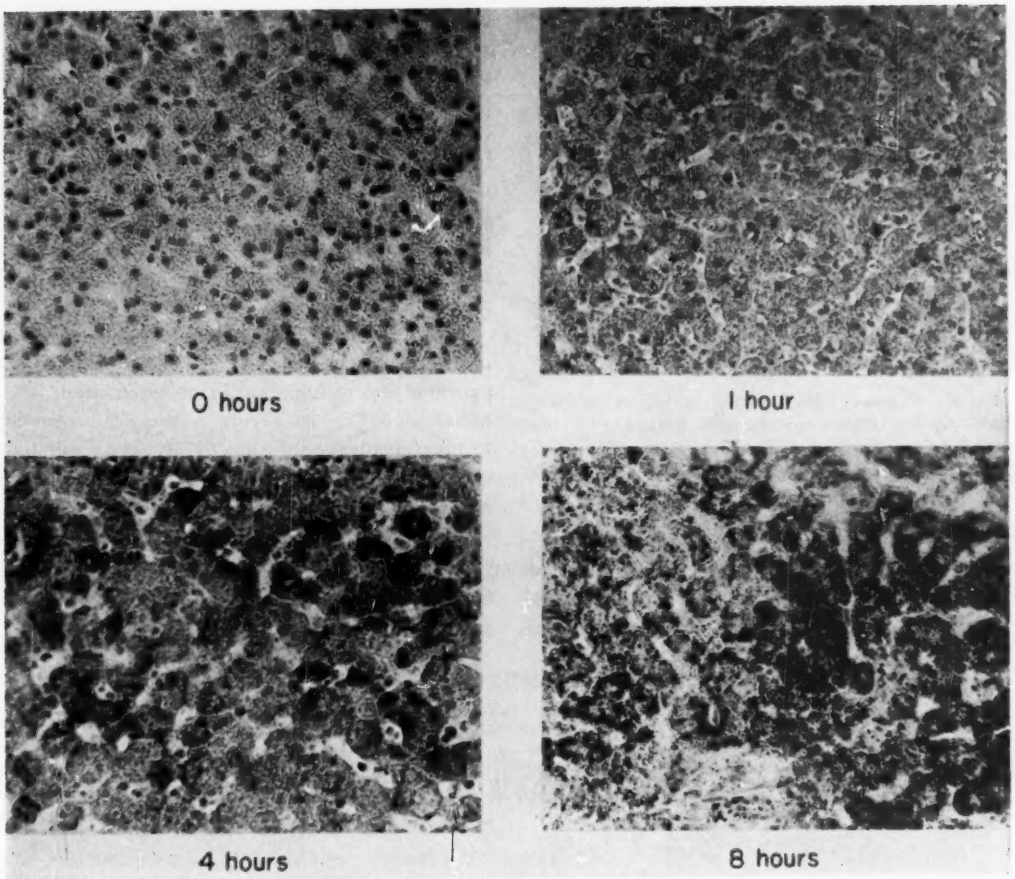

FIG. 3. HISTOLOGIC SECTIONS OF LIVER BIOPSIES TAKEN DURING THE COURSE OF THE EXPERIMENT ILLUSTRATED IN FIGURE 2 AT 0, 1, 4 AND 8 HOURS. The lipid staining material appears black in this reproduction (stained with Oil Red O).

very small changes. These were consistently positive over the 8-hour period, but the mean changes were not significantly different in control and experimental groups. The mean change for total cholesterol in the control group was + 8 per cent and in the experimental group + 12.4 per cent. Mean phospholipid change in the control group was + 10.3 per cent and in the experimental group + 6.9 per cent. Thus, norepinephrine did not appear to have an influence on these small changes, while it markedly affected triglyceride concentrations. Comparable increases in plasma FFA and liver triglyceride were achieved with the two concentrations of norepinephrine infused.

The changes in plasma total cholesterol levels during and after systemic infusion of norepinephrine were variable in the anesthetized, operated animals. In five experiments, changes in the plasma cholesterol levels 6 hours after the start of the infusion varied from + 13 to − 16 per cent, with a mean change of + 1 per cent. In three of these animals there were significant increases (12, 21 and 24 per cent, respectively) at some time in the period between 4 and 8 hours after the start of the infusion. Plasma cholesterol levels were determined at 24 hours (16 hours after termination of the infusion) in four animals. Two showed no rise; two showed elevations of 17 and 24 per cent, respectively. The variability in these responses may be due to the anesthetic or

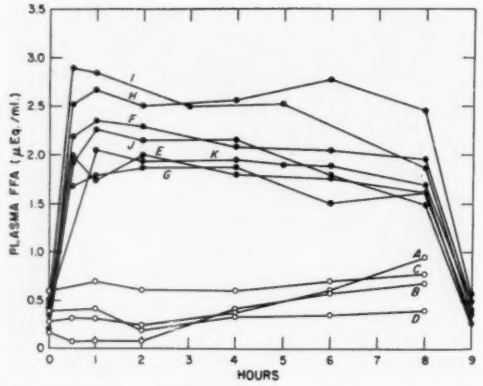

FIG. 4. PLASMA FFA CURVES OF SEVEN ANIMALS (●——●) RECEIVING CONTINUOUS 8-HOUR INFUSIONS OF NOREPINEPHRINE AND OF FOUR CONTROL ANIMALS (○——○) RECEIVING SALINE. The letters on each curve correspond to the same letters in Table I, which shows the corresponding liver lipid changes.

operative procedures, or both, including the handling of the liver. Two studies were done with an anesthetized dog *not* subjected to surgical biopsy until the end of the 8-hour infusion, the first using epinephrine, the second using norepinephrine. On both occasions the serum cholesterol at 8 hours was found to be elevated (35 and 29 per cent above control levels, respectively) and then fell toward normal at 24 hours. The timing of these responses differs notably from that seen when unanesthetized dogs were given epinephrine in oil, in which case there was no elevation at 6 hours but consistent elevations, averaging 28.6 per cent, at 24 hours (23).

Effect on liver lipids of 8-hour norepinephrine infusion into the portal vein. That the liver is a principal site of norepinephrine inactivation is established (24). In Figure 5, the FFA response to norepinephrine infusion (1 μg per kg per minute) via the portal vein is seen to be insignificant when compared with the FFA response to the same amount of norepinephrine infused into the femoral vein of the same dog. Failure of the FFA to increase during portal vein infusion was associated with failure of the mean blood pressure to rise sig-

TABLE I

Changes in liver lipid concentrations induced by norepinephrine infusion

Dog	Hour	Total cholesterol	Free cholesterol	Phospholipids	Triglycerides
		mg/g liver wet wt		*mg/g liver wet wt*	
Controls (saline infusion)					
A	0	1.99	1.77	27.3	4.7
	8	2.19	2.06	30.6	8.1
	Difference	0.20	0.29	3.3	3.4
B	0	2.02	1.89	27.5	4.2
	8	2.20	1.98	31.6	5.2
	Difference	0.08	0.09	4.1	1.0
C	0	2.09	1.88	30.3	3.4
	8	2.32	2.08	32.8	4.2
	Difference	0.23	0.20	2.5	0.8
D	0	2.79	2.41	33.6	8.1
	8	3.02	2.46	35.6	11.0
	Difference	0.23	0.05	2.0	2.9
	Mean increase in triglycerides: + 39%				
Norepinephrine (1 μg/kg/min by femoral vein infusion)					
E	0	2.48	2.10	33.5	7.6
	8	2.55	2.13	34.6	32.0
	Difference	0.07	0.03	1.1	24.4
F	0	2.38	2.08	30.8	3.0
	8	2.71	2.35	33.5	39.0
	Difference	0.33	0.27	2.7	36.0
G	0	3.15	2.90	34.8	4.1
	8	3.25	3.00	39.2	40.0
	Difference	0.10	0.10	4.4	35.9
	Mean increase in triglycerides: + 799%				

TABLE I—(*Continued*)

Dog	Hour	Total cholesterol	Free cholesterol	Phospholipids	Triglycerides
		mg/g liver wet w t		*mg/g liver wet wt*	
Norepinephrine (2 μg/kg/min by femoral vein infusion)					
H	0	2.50	2.33	30.6	3.5
	8	2.99	2.65	31.6	24.0
	Difference	0.49	0.32	1.0	30.5
I	0	1.94	1.89	27.3	5.3
	8	2.48	2.22	30.5	51.0
	Difference	0.54	0.33	3.2	45.7
J	0	2.05	1.85	29.3	2.5
	8	2.30	2.10	30.1	33.0
	Difference	0.25	0.25	0.8	30.5
K	0	2.92	2.63	29.9	18.0
	8	3.13	2.65	31.7	29.0
	Difference	0.21	0.02	1.8	11.0
	Mean increase in triglycerides: + 754%				
Norepinephrine (1 μg/kg/min by portal vein infusion)					
L	0	2.73	2.17	30.0	9.0
	8	3.02	2.19	31.0	9.5
	Difference	0.29	0.02	1.0	0.5
M	0	2.45	2.18	31.1	3.4
	8	2.84	2.51	34.9	3.4
	Difference	0.39	0.33	3.8	0.0
	Mean increase in triglycerides: + 2.8%				
Norepinephrine (2 μg/kg/min by portal vein infusion)					
N	0	3.04	2.33	30.9	13.0
	8	3.23	2.42	31.6	14.0
	Difference	0.19	0.09	0.7	1.0
O	0	2.86	2.18	31.3	18.0
	8	3.19	2.25	31.4	28.0
	Difference	0.33	0.07	0.1	10.0
	Mean increase in triglycerides: + 32%				

nificantly. When systemic infusion was started, both blood pressure and FFA levels rose. Figure 6 illustrates the differences in plasma FFA levels between dogs receiving an 8-hour norepinephrine infusion at a rate of 1 μg per kg per minute systemically and dogs receiving norepinephrine at the same rate via the portal vein. At the end of 8 hours, after liver biopsies were taken, the portal vein infusions were changed to systemic infusion to demonstrate that the dogs were capable of responding with an FFA increase. The liver lipid changes resulting from the infusions depicted in Figure 6 are included in Table I. There were no changes in the dogs receiving the portal vein infusions (L, M), but the triglyceride changes associated with the systemic infusions are great (E, G). In two additional experiments the rate of norepinephrine infusion into the portal vein was doubled (2 μg per kg per minute), and in these experiments rises in plasma FFA concentrations comparable to those seen with systemic infusion of 1 μg per kg per minute were seen, presumably because some of the infused norepinephrine escaped inactivation during the first passage through the liver. One of these animals (Dog O, Table I) showed a rise in liver triglyceride concentration, but the second animal (Dog N, Table I) failed to show any change in liver triglyceride concentration. It should be noted that both animals given the high rate of norepinephrine infusion intraportally died, for reasons unknown, within 6 hours after completion of the experiment.

Fatty acid composition of liver lipids, plasma FFA and adipose tissue before infusion of nor-

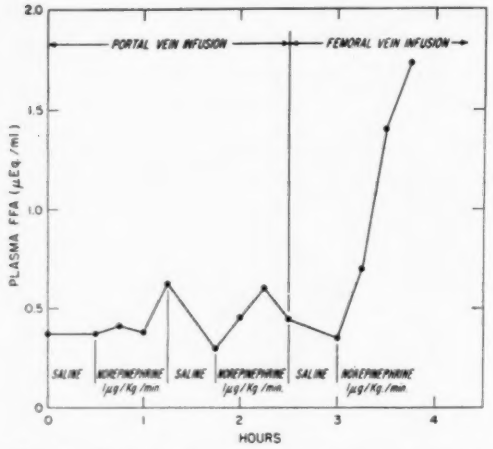

FIG. 5. COMPARISON IN THE SAME ANIMAL OF PLASMA FFA RESPONSE TO NOREPINEPHRINE INFUSION VIA THE PORTAL VEIN IN TWO SUCCESSIVE 45-MINUTE PERIODS, INTERRUPTED BY SALINE INFUSIONS, WITH PLASMA FFA RESPONSES TO THE SAME RATE OF NOREPINEPHRINE INFUSION DELIVERED VIA FEMORAL VEIN.

epinephrine, and of liver lipids and plasma FFA after infusion. In experiment F (see Table I and Figure 4), the fatty acid compositions of the liver lipid fractions, plasma FFA and adipose tissue were determined and are shown in Figure 7. While the triglyceride content of the liver increased from 3 to 39 mg per g liver, the decrease in linoleic acid at the end of 8 hours was only 18 per cent. That linoleic acid was not contributed

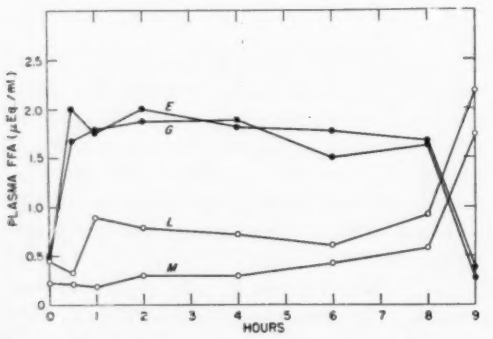

FIG. 6. COMPARISON BETWEEN PLASMA FFA RESPONSES IN TWO ANIMALS RECEIVING NOREPINEPHRINE VIA THE FEMORAL VEIN (●——●) AND THE PLASMA FFA RESPONSES IN TWO ANIMALS RECEIVING THE SAME RATE OF NOREPINEPHRINE INFUSION VIA THE PORTAL VEIN (○——○). The letters on each curve correspond to the same letters in Table I, which shows the corresponding liver lipid changes.

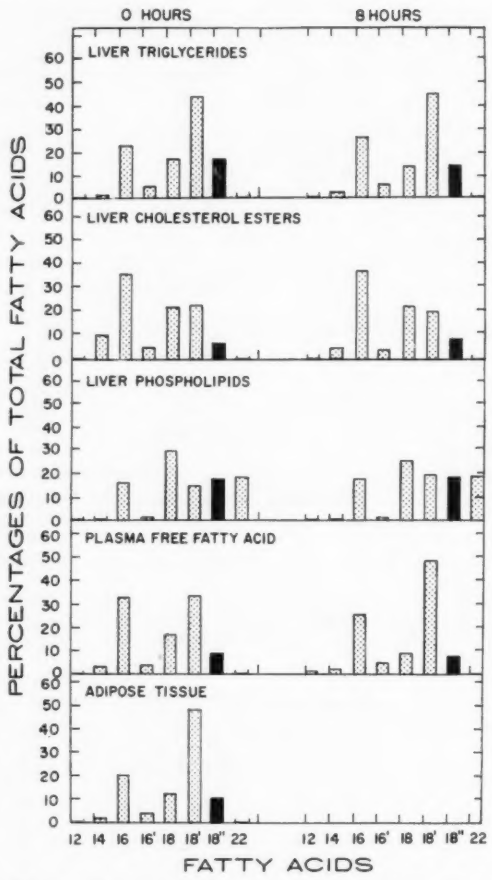

FIG. 7. FATTY ACID COMPOSITION OF ADIPOSE TISSUE, PLASMA FFA AND LIVER LIPID FRACTIONS AT ZERO TIME (LEFT) AND OF PLASMA FFA AND LIVER LIPID FRACTIONS AFTER 8 HOURS OF NOREPINEPHRINE INFUSION (RIGHT).

to triglyceride by transfer from phospholipid or cholesterol esters is apparent from examination of Table I and Figure 7. First, the absolute concentration of these fractions actually increased slightly, and second, the per cent linoleic acid in them, rather than decreasing, increased somewhat over zero time values. The fatty acid composition of the liver triglycerides, before and after norepinephrine, was similar to the composition of the fatty acids of adipose tissue as determined in a biopsy specimen taken from the mesenteric fat. The fatty acid composition of the plasma FFA after norepinephrine infusion also became almost identical to the composition of the adipose tissue triglycerides.

DISCUSSION

These experiments demonstrate that a sustained elevation of plasma FFA concentration, induced in this case by constant intravenous infusion of norepinephrine, is associated with a progressive, rapid increase in the level of triglycerides in the liver. The latter effect is related to either: *1*) the elevated FFA concentration in the plasma and saturation of the normal mechanism for handling FFA by the liver, *2*) liver damage from the experimental procedure in which the normal mechanism for handling FFA by the liver was compromised, *3*) a direct or indirect effect of norepinephrine on the liver, or *4*) a combination of any of the possibilities noted above. That the fatty liver was not caused by a direct effect of norepinephrine on liver metabolism is demonstrated by the portal vein infusion experiments in which norepinephrine failed to affect liver triglycerides except when plasma FFA was also affected. The possibility that the vasomotor effects of norepinephrine on hepatic circulation may have played a role in the development of the fatty liver cannot be ruled out by these experiments. That the experimental procedure, per se, independent of the norepinephrine effect, may have significantly damaged the liver seems unlikely, since no histopathological changes were noted in the livers of control animals and because of the highly significant differences in liver triglyceride observed between control and experimental animals. However, the possibility that the experimental procedure may have, in some unknown manner, restricted the normal capacity of the liver to handle FFA cannot be excluded.

The study of the fatty acid composition of the newly deposited triglycerides helps establish that they were not synthesized by the liver *de novo*, since the linoleic acid content increased in proportion to the over-all increase in triglyceride fatty acids (25). It has been shown that linoleic acid is not synthesized by mammalian liver (26) and rats, in particular, can be made deficient in linoleic acid (27). The fact that the proportion of linoleic acid in the hepatic triglycerides remains the same despite a large increase in the total hepatic triglycerides does not prove that all of the fatty acids are derived from the periphery under the influence of norepinephrine, but does prove that the fraction of fatty acids derived from the periphery probably remains the same under the influence of the hormone as in control conditions. Furthermore, the pattern of the plasma FFA during the infusion of norepinephrine was altered so as to become almost identical with the pattern of fatty acids in the adipose tissue and strikingly similar to the fatty acid pattern of the newly formed hepatic triglycerides. These results seem to justify the hypothesis that the most probable sequence of events is the following: mobilization of fatty acids from adipose tissue by norepinephrine, transport to the liver, and incorporation into liver triglycerides.

It has been established that an intact adrenal gland is essential for the production of certain kinds of fatty liver. This is true for the fatty liver due to phosphorus poisoning (1), prolonged fasting (2), exposure to cold (2), treatment with ethionine (3), and treatment with epinephrine (4). Recently it was shown directly that the ability of the animal to mobilize fatty acids from adipose tissue in response to epinephrine stimulation likewise depends upon the adrenal gland (28, 29) and possibly the pituitary gland (30) as well. It was suggested that the failure to induce fatty liver in the absence of the adrenal gland might be a reflection of this decreased ability of adipose tissue to respond in the animal with adrenocortical insufficiency. Also, it has been shown that adrenergic blocking agents inhibit the epinephrine-induced release of FFA from adipose tissue *in vitro* (31), and that they also reduce the triglyceride deposition in normal rat livers induced by carbon tetrachloride, ethionine and ethanol (25).

Putting the present studies into the context of available information concerning development of fatty liver, it is theoretically possible to propose two general categories, as indicated in Figure 8. On the one hand, fatty liver can be produced in the absence of hepatic damage if the rate at which fatty acids are brought to a normal liver exceeds its ability to metabolize them further, either by oxidation or by resecretion into the plasma as components of the lipoproteins. In the present experiments, there is no reason to believe that the liver was in any way damaged, unless the anesthesia or the surgical manipulation induced temporary derangement of function. On the other hand, when the liver is damaged or metabolically altered, there may be an inability to handle even

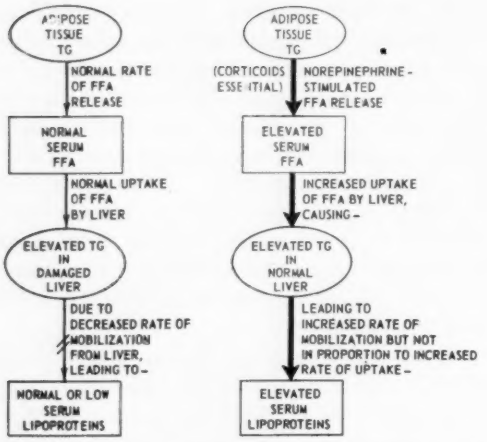
ADIPOSE TISSUE TG
NORMAL RATE OF FFA RELEASE
NORMAL SERUM FFA
NORMAL UPTAKE OF FFA BY LIVER
ELEVATED TG IN DAMAGED LIVER
DUE TO DECREASED RATE OF MOBILIZATION FROM LIVER, LEADING TO –
NORMAL OR LOW SERUM LIPOPROTEINS
ADIPOSE TISSUE TG
(CORTICOIDS ESSENTIAL)
NOREPINEPHRINE - STIMULATED FFA RELEASE
ELEVATED SERUM FFA
INCREASED UPTAKE OF FFA BY LIVER, CAUSING –
ELEVATED TG IN NORMAL LIVER
LEADING TO INCREASED RATE OF MOBILIZATION BUT NOT IN PROPORTION TO INCREASED RATE OF UPTAKE –
ELEVATED SERUM LIPOPROTEINS

FIG. 8. PROPOSED SCHEME SUMMARIZING THE ROLE OF PLASMA FFA IN THE DEVELOPMENT OF FATTY LIVER IN A NORMAL LIVER (RIGHT) AND IN A DAMAGED OR METABOLICALLY ALTERED LIVER (LEFT). Mobilization, as used in this scheme, includes oxidation to CO_2 or secretion into the plasma as component lipids of the lipoproteins, or both.

the normal flow of FFA from the depot. Hepatotoxic agents, such as carbon tetrachloride, may reduce the capacity of the liver to form and secrete lipoproteins or to oxidize fatty acids and thus lead to accumulation of liver fat. In some instances, of course, the fatty liver may be due to a combination of these circumstances, i.e., an increased rate of delivery of fatty acids to the liver and a decreased ability of the liver to handle them. This is not meant to imply that all fatty livers can be explained on this simple basis. For example, there is evidence to suggest that the fatty liver due to ingestion of alcohol may in part be the result of increased rates of fatty acid synthesis in the liver (32). However, the accumulated evidence supporting the importance of transport of fat from depot to liver, presumably as FFA, in the development of fatty liver from many causes is impressive.

SUMMARY

Sustained elevation of plasma FFA concentration in dogs, induced by infusion of norepinephrine into the femoral vein, was associated with a progressive and rapid rise in liver triglyceride. The same concentration of norepinephrine infused into the portal vein caused little elevation of plasma FFA and no rise in liver triglyceride. Studies of the fatty acid composition of the liver triglyceride, plasma FFA and adipose tissue triglyceride were consistent with the hypothesis that the newly formed liver triglyceride was synthesized from plasma FFA mobilized from adipose tissue. The role of plasma FFA in the development of fatty liver is discussed, and a scheme for the development of fatty liver under a variety of conditions is presented.

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THE RAPID INTRAVENOUS GLUCOSE TOLERANCE TEST IN PREGNANCY*

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Pregnancy may precipitate frank diabetes (1–5) or aggravate diabetes with regard to the clinical course and insulin requirements (6–10). Not all susceptible females are equally affected and some escape deterioration of carbohydrate metabolism during a particular pregnancy (10–14). The detrimental effects are frequently interrupted early in the puerperium (1, 2, 14–16). Consideration of the diabetogenic factors involved has focused attention on the contra-insulin effects of cortisone and growth hormone (2, 5, 17). Insulin degradation by one of the products of conception, the placenta (18), and insulin inactivation by plasma protein (19) represent other potential factors. The contra-insulin effects have been interpreted as a challenge to the insulin-producing capacity of the maternal pancreas (2, 3).

The limitations of the oral glucose tolerance test, particularly during pregnancy (20–23), cast doubt on interpretations indicating impaired tolerance in nondiabetic women (24–27). Intravenous tolerance tests have been inconclusive (28) with some values (29) indicating that glucose tolerance may improve during uncomplicated pregnancy. There is at present no convincing evidence of altered glucose tolerance in the nondiabetic pregnant female, despite the multiplicity of metabolic changes during pregnancy and the adverse effects upon the diabetic state. Failure to disclose significant alterations in tolerance may have resulted from: *1*) inadequacies of the oral glucose tolerance test because of the interaction between glucose absorption and glucose uptake by tissues; *2*) insensitivity of the intravenous test methods employed, since only a rough estimate of tolerance can be derived from the fall in blood glucose concentration within a preselected interval, usually 2 hours (30); or *3*) the absence of a net change in glucose tolerance during pregnancy. The last possibility could reflect a balance between the effects of increased insulin production and contra-insulin mechanisms. This possibility also offers a basis for the maintenance of tolerance during pregnancy in the nondiabetic and the deterioration of tolerance in the diabetic female, assuming a relative inability of the diabetic to meet a demand for more insulin.

This investigation was directed at revaluating the pattern of glucose tolerance during uncomplicated pregnancy in nondiabetic women. The slope of the tolerance curve after the injection of a standard dose of glucose served as the index of glucose tolerance. Previous experience with this technique has shown it to be sufficiently sensitive to reveal changes in glucose tolerance among nondiabetic male subjects on the basis of differences in age (30).

EXPERIMENTAL METHODS

Subject selection. One hundred sixteen ambulatory female subjects, aged 16 to 41 years, were screened by a detailed history, physical examination, urinalysis and fasting blood glucose determination. Subjects presenting any of the following were excluded from the study: *1*) a history or evidence of diabetes or glycosuria; *2*) a family history of diabetes; *3*) a suspicious obstetrical history including babies of excessive weight (exceeding 4.0 kg) and unexplained stillbirths; *4*) alcoholism, hepatomegaly, cirrhosis or other liver disease; *5*) thyroid or other endocrine disturbance; *6*) cardiac decompensation, edema, toxemia and abnormalities of pregnancy; *7*) gastrointestinal upsets; *8*) the taking of steroid drugs (all other medication was omitted for 24 hours before the test); and *9*) infection, temperature elevation, or trauma (including surgical) within 1 week of the test, except for the postpartum group which was tested after the third postpartum day. The previous diet provided 2,000 calories daily for at least 1 week, again with the exception of

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TABLE I

Subject characteristics

Variable		Non-pregnant	Pregnant (trimester)				Post-partum
			First	Second	Third	All	
N		30	21	20	20	61	25
Age (*yrs*)	Mean	26.0	24.3	24.4	23.2	24.0	26.4
	Range	19–35	17–34	17–34	17–37	17–37	16–41
Height (*cm*)	Mean	162.7	160.0	159.6	159.2	159.6	156.4
	Range	150–179	153–173	152–176	150–173	150–176	147–167
Weight (*kg*)	Mean	59.7	60.4	59.0	65.5	61.6	58.2
	SE of mean	2.3	2.5	2.3	2.7	1.5	1.9
	Range	47–95	48–101	45–89	49–100	45–101	44–86
Fasting blood glucose (*mg/100 ml*)	Mean	65.9	61.3	59.1	59.6	60.0	56.1
	SE of mean	1.2	1.5	1.6	1.9	1.0	2.0
	Range	52–84	50–75	45–77	44–78	44–78	40–91

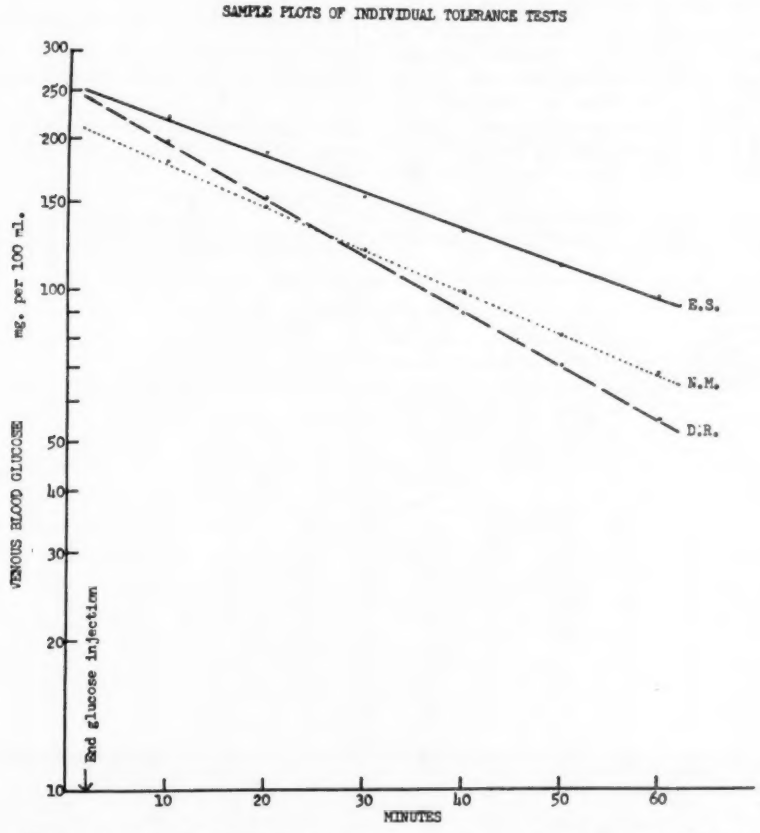

FIG. 1. LOG GLUCOSE CONCENTRATION IN VENOUS BLOOD (MILLIGRAMS PER 100 ML) PLOTTED AGAINST TIME (MINUTES) FOR INDIVIDUALS SELECTED FROM THE FOLLOWING GROUPS: NONPREGNANT (SOLID LINE), THIRD TRIMESTER (DOTTED LINE), AND FIRST TRIMESTER (DASHED LINE).

the postpartum group, in which the minimal period was 3 days.

Experimental procedure. With the subject in the basal state, a modified Lindeman needle was inserted into an antecubital vein 20 minutes before the test and left in place until completion of the procedure. Patency of the needle was maintained by heparinization of the stylus. This needle was used only for the subsequent drawing of blood specimens without a tourniquet (31) and for obtaining a fasting sample a few minutes before zero time. The latter was recorded at the beginning of the injection of 50 ml of 50 per cent glucose in water into a vein of the opposite arm over a period of 2 minutes.

Blood samples were collected at 10 minutes and at 10-minute intervals thereafter until 60 minutes. The blood was immediately placed in dried heparin-fluoride tubes and analyzed on the day of the test by the Nelson-Somogyi method (32). Determinations were in duplicate and read on a Klett-Summerson photoelectric colorimeter.

Data analysis. The observations obtained between 10 and 60 minutes for each glucose tolerance test (GTT) were fitted to the equation, $\log_e y = \log_e A - kt$, where y is the blood glucose concentration in milligrams per 100 ml, A is the intercept with the vertical axis, and t is the lapsed time in minutes. The value of k, the index of tolerance for the study, was computed for each subject from the visually fitted slope to a plot of the log glucose concentration against time.[1] Glucose spaces were derived by extrapolating the GTT plots to the intercept at zero time without applying a correction factor (33) for the concentration of solids in whole blood.[2] Changes in glucose tolerance were evaluated by comparing the k values of the following groups: nonpregnant, nonmenstruating (30 subjects); pregnant, first trimester (21 subjects); pregnant, second trimester (20 subjects); pregnant, third trimester (20 subjects); pregnant, total (61 subjects); and postpartum, fourth through the sixth day (25 subjects).

RESULTS

Reliability of methods. The standard error of estimate of repeated glucose determinations on a single blood filtrate was ± 0.61 mg per 100 ml (N = 55, mean glucose concentration = 122 mg per 100 ml). Corresponding values for duplicate filtrates prepared from the same blood specimen were ± 0.92 mg per 100 ml (N = 100, mean glucose concentration = 119 mg per 100 ml).

General description of results. No significant differences in age were found among the subject

[1] The derivation of k by least squares has been compared with graphic estimates obtained from visual fits (30). The results were in sufficiently close agreement for the GTT (R = 0.96) to substantiate the use of the visual method.

[2] Glucose space (liters) = injected dose (25 g) / (0 time concentration − fasting concentration (g/L))

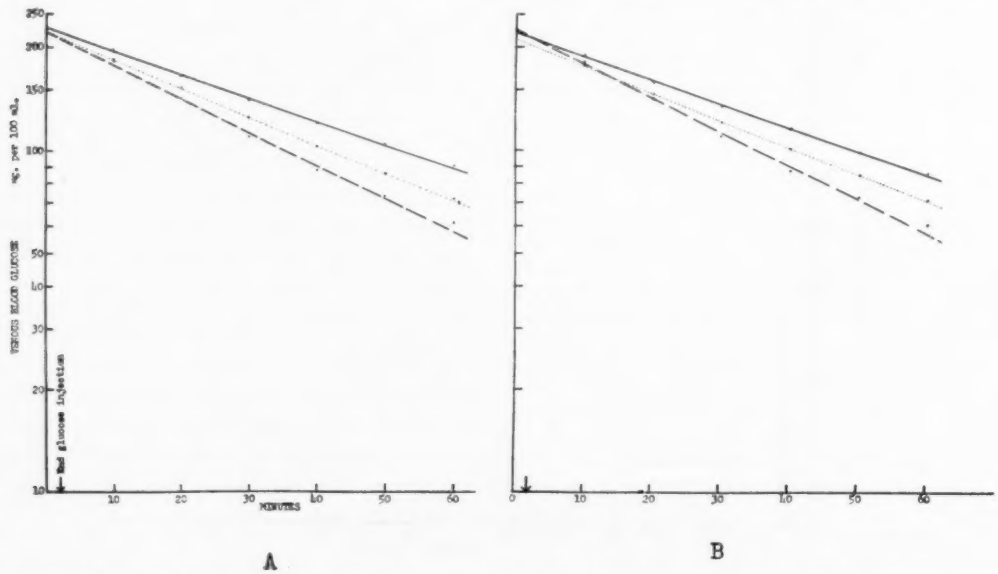

FIG. 2. CHANGES IN THE RAPID INTRAVENOUS GLUCOSE TOLERANCE TEST IN PREGNANCY. Logs of average venous blood sugar values (milligrams per 100 ml) for each group plotted against time (minutes) after the intravenous injection of 25 g of glucose. A. Nonpregnant (solid line), second trimester (dotted line), first trimester (dashed line). B. Postpartum (solid line), third trimester (dotted line), first trimester (dashed line).

TABLE II

Nonpregnant women of childbearing age (30 subjects)

Subject	Age	Ht	Wt	Blood levels after 25 g glucose i.v. (mg/100 ml at time in min) 0	10	20	30	40	50	60	k	Glucose volume
	yrs	*cm*	*kg*								*%/min*	*L*
M.J.	25	170	94	66	180	153	130	117	100	89	1.50	17.6
J.K.	21	161	47	67	231	186	165	144	127	112	1.65	12.3
B.D.	33	166	51	69	200	167	144	124	109	92	1.64	15.0
D.J.V.	30	164	82	80	223	198	173	156	140	133	1.40	14.7
P.G.	19	166	50	69	218	196	164	145	130	108	1.18	15.2
P.P.	23	157	60	70	197	170	147	131	116	100	1.31	16.5
N.L.	35	159	49	60	185	149	126	108	100	88	1.81	15.4
E.S.	29	159	51	64	222	189	152	130	113	99	1.76	12.4
H.R.	28	179	63	66	182	151	123	109	96	84	1.73	16.9
L.S.	20	164	50	66	224	117	155	136	124	112	1.50	14.3
G.C.	24	164	75	75	186	167	150	136	123	116	1.07	18.9
J.O.	29	172	56	70	188	159	140	129	115	99	1.35	17.4
P.C.	25	161	52	64	201	173	161	145	134	121	1.39	15.7
O'S.J.	26	166	62	64	194	150	127	104	83	72	2.04	14.5
E.F.	29	155	56	68	215	174	155	150	136	125	1.07	16.1
M.K.	23	154	56	60	200	172	139	122	112	94	1.57	14.5
D.L.	23	155	49	62	237	204	181	159	139	119	1.33	12.2
L.R.	32	150	48	60	198	151	119	92	78	59	2.47	13.3
B.M.	27	159	50	60	239	162	130	103	85	80	2.25	12.7
C.C.	23	167	59	52	170	150	133	115	102	92	1.25	18.1
M.P.	26	172	66	65	181	126	97	79	59	49	2.73	15.2
E.W.	22	154	49	62	188	150	124	97	80	66	2.14	14.6
M.V.	32	157	61	65	162	138	125	104	92	89	1.43	20.8
J.B.	21	172	60	64	194	145	114	92	80	70	2.09	15.6
R.T.	25	169	58	62	150	131	112	88	74	72	1.59	15.8
N.S	25	161	59	66	204	158	133	110	91	80	1.90	14.7
I.I.	30	162	64	57	208	171	147	129	115	94	1.54	13.5
Q.N.	23	157	56	71	231	177	140	124	111	88	1.78	14.1
G.J.	24	169	64	69	182	139	108	80	71	62	2.44	17.3
T.J.	27	160	95	84	205	177	155	141	126	116	1.28	17.6
Mean	26.0	162.7	59.7	65.9	199.8	163.7	139.0	120.0	105.4	92.7	1.67	15.4

TABLE III

Pregnant women in the first trimester (21 subjects)

Subject	Age	Ht	Wt	Blood levels after 25 g glucose i.v. (mg/100 ml at time in min) 0	10	20	30	40	50	60	k	Glucose volume
	yrs	*cm*	*kg*								*%/min*	*L*
E.K.	32	161	52	61	180	106	75	58	46	41	3.45	13.1
S.D.	34	157	56	60	180	152	126	115	105	93	1.31	14.0
G.G	21	173	71	62	154	117	85	70	59	53	2.62	18.1
R.S.	20	157	48	50	179	113	81	42	35	27	3.38	14.6
D.C.	26	161	62	69	187	129	102	73	69	59	2.56	16.1
E.M.	26	170	66	62	170	145	79	76	65	56	2.46	17.1
J.P.	24	165	59	67	162	105	64	55	43	41	3.23	18.9
G.M.	21	154	61	55	165	140	118	100	99	71	1.73	17.7
I.V.	18	155	49	57	197	161	128	95	75	58	2.44	12.9
S.V.	23	166	53	60	208	178	128	102	99	63	2.38	12.3
T.C.	28	156	57	62	172	145	115	106	95	88	1.70	17.7
C.R.	20	166	59	71	186	138	100	77	58	53	3.20	14.1
M.P.	23	165	72	64	184	150	123	106	97	87	1.85	17.2
D.G.	24	157	66	75	180	156	138	115	102	91	2.99	18.9
P.M.	31	155	101	71	164	132	106	87	74	68	2.33	17.4
L.S.	22	155	56	57	185	143	101	86	75	72	2.53	14.3
M.McM.	29	161	62	58	184	156	138	123	110	106	1.36	16.3
D.R.	27	153	50	57	198	153	117	89	72	56	2.53	12.9
A.A.	21	157	55	50	179	142	107	84	72	54	1.91	17.4
F.M.	23	155	62	66	230	180	156	132	113	90	1.71	12.3
A.R.	17	161	52	54	180	129	96	60	48	45	3.15	13.2
Mean	24.3	160.0	60.4	61.3	182.1	141.4	108.7	85.5	76.7	65.3	2.42	15.6

TABLE IV

Pregnant women in the second trimester (20 subjects)

Subject	Age	Ht	Wt	Blood levels after 25 g glucose i.v. (mg/100 ml at time in min) 0	10	20	30	40	50	60	k	Glucose volume
	yrs	*cm*	*kg*								*%/min*	*L*
C.R.	32	152	49	77	231	184	171	148	129	109	1.27	13.0
M.G.	28	156	54	71	216	184	160	139	117	101	1.43	14.5
D.B.	28	162	66	61	178	135	105	77	63	49	2.52	16.3
R.R.	20	162	75	63	175	142	110	87	73	61	2.19	16.7
H.A.	34	162	71	65	182	155	128	112	93	78	1.68	18.3
I.C.	21	155	61	54	190	153	119	89	72	58	2.42	12.9
C.D'A.	22	157	60	55	189	153	131	110	93	79	1.75	15.2
M.O'F.	22	166	56	59	149	145	125	106	94	78	1.45	19.1
J.R.	25	173	54	59	195	157	133	102	88	77	2.03	13.8
T.K.	27	157	56	51	169	129	106	88	70	61	2.14	16.5
B.L.	23	176	47	53	187	149	116	85	73	60	2.40	13.4
E.S.	22	159	61	56	164	136	121	103	91	75	1.50	18.8
C.L.	21	154	60	53	217	176	147	118	101	88	1.93	12.0
A.R.	28	161	58	68	187	165	147	130	115	103	1.29	17.5
G.R.	17	157	60	56	160	132	104	87	75	63	2.15	27.2
M.F.	20	162	58	57	169	131	102	82	71	63	2.38	16.1
H.M.	21	157	45	56	198	168	144	122	104	88	1.60	14.3
R.B.	23	153	48	62	200	155	120	94	73	58	2.50	12.8
S.G.	28	155	51	61	203	156	122	102	87	76	1.76	13.4
O.K.	26	155	89	45	160	130	102	76	65	57	2.05	10.4
Mean	24.4	159.6	59.0	59.1	186.0	151.8	125.7	102.9	87.4	74.1	1.92	15.6

groups (Table I). A small decrease in weight occurred ($p = < 0.05$) in the postpartum subjects compared with the third trimester group. This is an anticipated finding in a random sample of women after childbirth. Fasting blood sugar concentrations throughout pregnancy and the postpartum period showed small, but statistically significant, decreases from the nonpregnant level.

A linear relationship was obtained when the log glucose concentration for each test was plotted

TABLE V

Pregnant women in the third trimester (20 subjects)

Subject	Age	Ht	Wt	Blood levels after 25 g glucose i.v. (mg/100 ml at time in min) 0	10	20	30	40	50	60	k	Glucose volume
	yrs	*cm*	*kg*								*%/min*	*L*
M.C.	18	155	56	54	204	164	132	100	79	71	2.12	12.8
D.C.	18	159	59	61	206	161	130	109	91	70	2.07	13.2
A.H.	19	164	100	67	156	135	114	103	92	82	1.41	23.2
R.H.	23	159	59	64	213	164	123	94	73	60	2.67	11.6
D.L.	23	167	66	61	155	130	103	80	62	49	2.40	18.7
A.C.	17	150	49	64	203	163	130	104	84	69	2.21	13.2
B.R.	25	154	56	78	196	162	136	119	102	90	1.68	18.1
Z.B.	19	156	66	61	175	145	117	95	78	64	2.14	16.3
A.L.	37	156	76	55	171	143	116	103	88	74	1.75	17.4
G.L.	30	157	80	63	140	119	104	94	89	78	1.24	27.2
N.M.	22	156	62	51	182	146	121	100	82	70	1.94	14.9
A.C.	28	173	50	62	206	171	150	131	112	96	1.51	14.2
D.G.	21	164	79	66	149	125	105	85	72	66	2.40	22.5
R.M.	26	159	68	56	140	119	90	76	66	53	2.00	21.6
E.G.	25	159	68	44	164	132	117	106	97	82	1.35	18.3
B.C.	19	166	61	48	149	117	101	79	67	59	1.98	19.2
H.M.	23	162	59	49	170	139	121	104	88	80	1.62	17.0
J.C.	22	153	60	67	215	168	137	116	91	74	2.87	13.3
M.R.	17	157	61	68	209	173	150	130	112	97	1.53	14.8
E.D.	32	157	75	53	166	139	121	109	95	83	1.37	18.4
Mean	23.2	159.2	65.5	59.6	178.5	145.8	120.9	101.9	86.0	73.4	1.91	17.3

TABLE VI

Women in the first postpartum week (25 subjects)

Subject	Age	Ht	Wt	Blood levels after 25 g glucose i.v. (mg/100 ml at time in min) 0	10	20	30	40	50	60	k	Glucose volume
	yrs	*cm*	*kg*								*%/min*	*L*
R.B.	23	149	48	54	203	150	122	96	71	61	2.45	12.5
F.F.	32	157	62	52	197	173	144	126	116	112	1.32	14.7
M.N.	23	153	71	73	196	155	140	115	113	103	1.46	17.2
E.K.	38	155	75	56	168	146	128	110	95	79	1.44	18.4
B.C.	19	166	54	56	191	160	148	137	125	116	1.05	16.1
M.D.	25	165	58	49	182	145	115	91	80	71	2.14	14.8
J.C.	22	150	54	91	218	166	124	103	80	71	2.46	13.6
R.M.	20	159	57	52	192	166	136	133	101	85	1.52	15.2
A.P.	41	165	63	53	164	140	127	114	97	82	1.25	18.9
A.R.	20	155	59	55	194	160	130	115	97	84	1.70	14.8
I.D.	34	153	61	50	174	150	128	112	95	76	1.44	16.8
M.M.	31	167	52	55	178	119	108	83	72	53	2.15	16.7
M.N.	22	157	51	55	188	161	137	128	113	104	1.23	16.2
I.P.	24	150	44	47	175	128	102	92	77	67	1.53	18.2
O.R.	19	157	56	66	185	156	127	106	96	78	1.79	15.5
H.C.	38	153	57	54	175	149	138	112	92	86	1.51	16.9
L.D.	20	153	54	47	191	173	157	139	120	115	1.09	14.4
C.C.	25	153	51	53	214	166	155	134	126	112	1.21	15.6
G.D.	24	160	69	56	177	139	116	94	79	71	1.97	15.9
M.P.	29	155	59	40	206	156	140	120	103	94	1.57	14.2
V.F.	27	147	59	56	222	192	171	157	141	124	1.16	12.9
V.R.	16	156	48	48	200	165	140	114	101	88	1.71	13.4
B.E.	31	150	48	70	220	186	160	140	122	103	1.51	13.7
S.J.	23	167	60	56	186	168	146	136	120	113	1.09	16.9
K.F.	35	159	86	58	161	115	102	83	82	74	1.68	19.7
Mean	26.4	156.4	58.2	56.1	190.3	155.4	133.6	115.6	100.6	88.9	1.58	15.7

from 10 to 60 minutes. There was a tendency for small upward deviations at the 10- and 60-minute points. Figure 1 shows individual log plots selected from the nonpregnant, first trimester, and third trimester groups. Log plots of the mean glucose values for five groups of subjects are separated into Figures 2A and 2B because of the nearly identical curves in the second and third trimester and the nonpregnant and postpartum groups, respectively. The rate of decline of blood glucose concentration appears greatest in the first trimester of pregnancy and recedes to an intermediate level in the second trimester. No change occurred between the second and third trimesters. During the first postpartum week the rate returns to that of the nonpregnant controls.

The slope, k, computed for each subject's GTT (Tables II–VI), provided numerical data for estimates of the variability within groups. Table VII summarizes the k values. Average values of k, expressed as per cent per minute, were as follows for each subject group: nonpregnant, 1.67;

TABLE VII

*Rate (k) of disappearance of glucose from venous blood after intravenous injection of 25 g glucose and extracellular distribution volume of glucose**

		Non-pregnant	Pregnant (trimester) First	Second	Third	All	Post-partum
N		30	21	20	20	61	25
k (*%/min*)	Mean	1.67	2.42	1.92	1.91	2.09	1.58
	SE of mean	0.08	0.14	0.09	0.10	0.07	0.08
Glucose volume (*L*)	Mean	15.4	15.6	15.6	17.3		15.7
	SE of mean	0.4	0.5	0.8	0.9		0.4

* Glucose volume = dose administered/blood glucose concentration at zero time − fasting concentration. No correction was made for concentration of water in whole blood.

first trimester, 2.42; second trimester, 1.92; third trimester, 1.91; and postpartum, 1.58. The overall effect of pregnancy, combining the three trimesters, was an increase in k from the nonpregnant level of 1.67 to 2.09.[3]

Extrapolated, extracellular distribution volumes of glucose were comparable in all groups, except the third trimester (Table VII). The volume increase of 1.9 ± 1.0 L (difference in means $\pm$ standard error of the difference) in the third trimester over the nonpregnant group was significant at $p = < 0.05$.

DISCUSSION

Subjects, activity and diet. Differences in age (30), activity (34) and diet (35) influence glucose tolerance. The age range was comparable in all groups. However, the postpartum group underwent the stress of childbirth (36), including 1 day of restricted food intake. Although not confined to bed, they were less active physically. These circumstances tend to depress glucose tolerance, and may have contributed to the fall in k during the postpartum period.

The tolerance equation in pregnancy. The glucose tolerance equation, $y = Ae^{-kt}$, graphically applied in the log form, $\log_e y = \log_e A - kt$, gathers the experimental, absolute glucose levels of each test and expresses them as a single derived value in the rate constant k (30, 37–39). This method has both the convenience and the limitations of a simple analytical curve used as a graduation device for expressing differences in the rate of disappearance of glucose. The rate constant is closely correlated with glucose clearance from the blood. Its reproducibility has been confirmed and adjustment of the glucose load to body size is unnecessary (30, 40). Comparable values of k in male subjects (30) are in close agreement with those of the nonpregnant female group, being 1.68 ± 0.9 and 1.67 ± 0.08, respectively. Cleempoel, Conard and Bastenie (41) reported normal values at 1.80 ± 0.16.

In vitro perfusion studies with nonmetabolized, glucose-like molecules (sorbitol) indicate rapid, extracellular equilibration, occurring well within 10 minutes (42). Generally, the phase of extracellular mixing in the GTT is likewise sufficiently completed by 10 minutes after the glucose injection to be indistinguishable from the body of the tolerance curve derived from venous blood samples. Interaction of the mixing phase with k is suggested in some individual curves by slight upward deflection of the 10-minute glucose values. Simultaneous estimates of the thiocyanate and glucose spaces, using the absolute glucose tolerance equation for estimates of the latter, have been in close agreement (39). Urinary excretion of glucose within 1 hour after an intravenous dose of 25 g was measured in 10 pregnant and 6 nonpregnant female subjects. The mean glucose excretion was 0.90 g, or 3.6 per cent of the dose, during pregnancy, and 0.80 g, or 3.2 per cent of the dose, in the nonpregnant state. Johnson and Bonsnes (29) also found the average urinary excretion of glucose after the intravenous injection of 25 g to be substantially the same in pregnant subjects (7.2 per cent) as in nonpregnant controls (6.8 per cent). These amounts represent a small fraction of the administered glucose load. The urinary losses occur during the early part of the tolerance curve when blood glucose levels and renal tubular loads are high and may, as pointed out by West and Wood (40), contribute to the early, slightly upward deviation of the tolerance curve from linearity. The latter part of the GTT curve involves an interaction with glucose release from the liver (43) which, in turn, probably accounts for late, upward nonlinearity, particularly when the curve is at or below fasting levels.

Greville (44) and Hlad, Elrick and Witten (45) computed an asymptotic value of blood sugar concentration, c, using the equation $\log_e(y - c) = \log_e A - kt$. This method partitions the expression of tolerance test data between the rate constant and the asymptote, since both terms are derived from the same set of experimental points.

[3] Differences in the mean values of k were significant at $p < 0.01$ between pairs of the following groups: nonpregnant and first trimester; first trimester and second trimester; and nonpregnant and the total of all trimesters. Differences in k were significant at $p < 0.05$ between the following paired groups: combined second and third trimesters and nonpregnant; and third trimester and postpartum. When regrouped into three classes for an analysis of variance—*A*) nonpregnant, *B*) first trimester, and *C*) second trimester—the differences in k between classes were significant at the 0.001 point. Combination of the second and third trimesters in class C, or substitution of the third for the second trimester group in class C or the postpartum for the nonpregnant group in class A, resulted in similar statistical estimates.

All k values are thereby rendered more nearly alike, and differences in glucose tolerance are projected into the asymptote. Recalculations, by this method, of the data from both the present study and a comparable, previously published report involving age differences in k (30) resulted in qualitative conclusions regarding glucose tolerance which were like those obtained without the asymptote (asymptote = zero).

The substitution of the fasting blood sugar level for a computed asymptote (46, 47) has been critically reviewed by Ikkos and Luft (48) and by West and Wood (40).

Changes in k during pregnancy. The index of tolerance was increased throughout pregnancy, but mainly in the first trimester. There is no evidence supporting an augmented response to insulin during pregnancy, other than some uncertainties pertaining to the rapidly growing conceptual products and the elevated metabolic rate (49). While the increased insulin effect indicated by k may have resulted from a greater release of insulin, other factors are involved. Growth and redistribution of body fluids occur as pregnancy progresses (50-52). Glucose crosses the placenta (53) and is utilized by the fetus (54). Chesley, Valenti and Uichanco estimated the total water gain of pregnancy as 6.3 L and the average gain in extracellular fluid (sucrose space) as 3.95 L, with 2.65 L as the maternal fraction (50). The increment of extracellular fluid was taken as 3.8 L by Seitchik and Alper (55), with 2.5 L for the maternal fraction. By comparison, the increment in glucose space, measured from the nonpregnant state to the third trimester, is 1.9 ± 1.0 L. While this figure is in fairly good agreement with estimates of the maternal fraction, it must be extended to its upper error range (2 standard errors) to approach other estimates of total extracellular fluid gain. However, of the 20 subjects in the third trimester, 11 were at less than 32 weeks' duration of pregnancy, 3 were beyond 36 weeks, and only 1 had reached 39 weeks. This distribution does not represent full-term growth and fluid attainment. Furthermore, failure to correct for hemodilution, reported with advancing pregnancy (52), may have introduced an underestimate of the increase in glucose volume. The effects of increasing cell mass and body fluid are minimal in the first trimester and progressively reach a maximum in later pregnancy. In contrast, the pattern of k changes (Table VII) proceeds in the opposite direction. This trend is not attributable to progressively increasing distribution volumes, although the latter probably contribute to the over-all elevation of k in pregnancy.

Hyperinsulinism of pregnancy. Simultaneous injection of glucose and insulin results in marked elevation of k (30). High k values have been observed in association with carcinoma of the pancreatic islets (46), presumably due to increased levels of endogenous insulin. The increased levels of k, particularly in early pregnancy, indicate a state of mild hyperinsulinism, possibly secondary to chronic pancreatic stimulation.

Welsh and Sims (56) found higher levels of plasma insulin-like activity in some of their pregnant subjects than in nonpregnant controls. Hoet and Lukens (2) have emphasized the hypertrophy and hyperplasia of the islets of Langerhans observed in the human and animal maternal pancreas during pregnancy, with marked regression of these changes in animals within 4 days after the birth of a litter.

SUMMARY

Glucose tolerance in pregnancy was studied by the intravenous glucose tolerance test under standardized conditions, using 25 g of glucose and venous blood samples. The rate of fall, k, obtained from a visual fit of the experimental sugar levels between 10 and 60 minutes to the equation $\log_e y = \log_e A - kt$, served as the index of tolerance. A total of 116 healthy women was tested, including 30 nonpregnant, 25 postpartum, and 61 subjects with uncomplicated pregnancies. There was a significant elevation in k during pregnancy, particularly in the first trimester, with a prompt return to nonpregnant levels in the postpartum period. The glucose space was slightly increased in the third trimester. It is proposed that k elevations may result from both mild hyperinsulinism and increased distribution volumes in pregnancy.

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IMMUNOLOGICAL SPECIFICITY OF HUMAN INSULIN: APPLICATION TO IMMUNOASSAY OF INSULIN

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Previous investigations have revealed evidence for species-specificity among various mammalian insulins in immunologic reactions (1–5). For this reason, immunoassay of plasma insulin (6–10) in any species generally requires that the insulin employed in standard solutions be derived from the same species. Immunoassay of plasma insulin in man has been carried out by comparing the effects of unknown plasma samples with those of standard solutions of human insulin in their ability to inhibit competitively the binding of I^{131}-labeled beef insulin by guinea pig antibodies to beef insulin (7–10). Since supplies of human insulin are relatively scarce, the immunoassay of endogenous plasma insulin in man would be expedited if an animal insulin in more plentiful supply could be substituted as a standard for human insulin. Insulins from four different ungulate species (beef, hog, sheep, horse) have been found to react quantitatively with human antiserums to beef-pork insulin in a manner that could be correlated (5) with the similarity or dissimilarity of the amino acid sequences as elucidated by Harris, Sanger and Naughton (11). Of these four insulins, pork insulin (11) resembles human insulin (12) most closely in amino acid sequence, differing only in the C-terminal residue of the B chain (alanine in pork insulin, threonine in human insulin). The other animal insulins differ from human insulin *also* in one or more of the 8 to 10 positions of the A chain. It seemed worthwhile, therefore, to examine various antiserums for similarity of reaction of pork and human insulin. The present communication describes insulin antiserums obtained from guinea pigs immunized with crystalline pork insulin that react identically with human insulin and pork insulin in respect to the competitive inhibition of binding of pork insulin-I^{131}.

METHODS

Guinea pigs were injected subcutaneously with approximately 5 U of crystalline pork insulin (Lilly, lot no. 499667) or crystalline beef insulin (Lilly, lot no. 535664) emulsified in 0.2 ml mannide monooleate or 10 U of protamine zinc beef insulin (Squibb) without adjuvant at intervals of 2 to 10 weeks to a total of 4 to 7 doses. Approximately 10 ml heparinized blood was taken by cardiac puncture about 2 weeks after insulin injections. Hypoglycemic reactions were virtually obviated by sprinkling table sugar over the chow and by adding glucose to the drinking water for 24 hours after insulin injections.

Human antiserums to insulin were obtained from insulin-resistant and nonresistant diabetic subjects treated with commercial mixtures of beef-pork insulin. Serums were taken at least 24 hours after the last injection of insulin and allowed to stand at 4° C for at least 8 weeks to permit destruction of any residual animal insulin by the plasma (10, 13).

All dilutions of antiserum and insulin were prepared in veronal buffer, ionic strength 0.1, pH 8.6, containing 0.25 per cent human serum albumin, to prevent losses of antibody or of insulin by adsorption onto glassware. When prepared as described, antiserums could be maintained at dilutions of 1:200 in the frozen state for many months without detectable loss of potency.

Solutions of beef and pork insulin were made from the crystalline preparations maintained in a desiccator at 4° C since receipt. These had been assayed at 27 to 29 U per mg.

For the present study, human insulin solutions were made from two different preparations. *1) "Fisher human insulin,"* a dense powdery preparation, was supplied to us in 1958 by Dr. A. M. Fisher, Connaught Laboratories, Toronto. This preparation had been assayed at 6.8 U per mg in 1956 but was thought possibly to have decreased slightly in hormonal potency by 1959. Since several crystalline animal insulins obtained from Dr. Fisher were estimated at about 22 U per mg, we had tentatively assigned a value of 6.0 U per mg to the human insulin, based on a reference standard of 22 U per mg for *pure* insulin (10). This material was tested by immunoassay in 1959 against a preparation of human insulin, obtained from Dr. F. Tietze (through the courtesy of Dr. J. Field, NIH), which had recently been assayed in the Lilly Research Laboratories. The assigned value of 6.0 U per mg for the Fisher insulin, referred to a standard of 22 U per mg for pure insulin, agreed excellently with the recently standardized Tietze insulin and was therefore accepted as indicating an insulin content of 6.0 U per mg/22 U per mg = 27.3 per cent insulin by weight (10). All concentrations reported here for this preparation are based

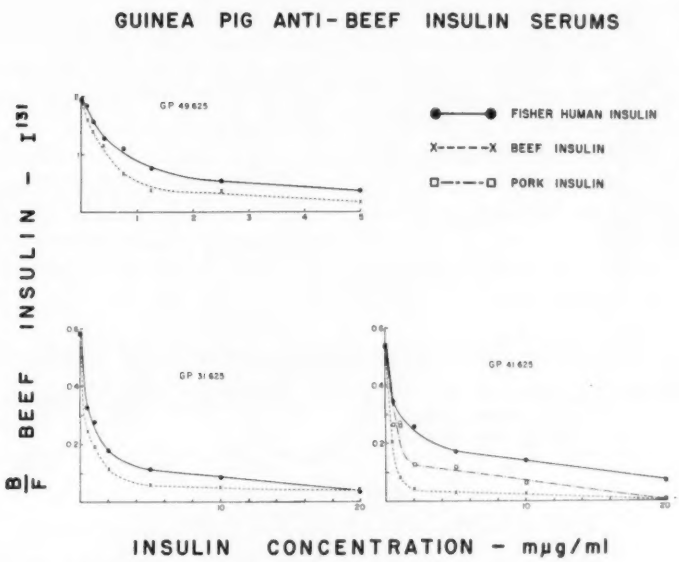

FIG. 1. RATIO OF ANTIBODY-BOUND BEEF INSULIN-I^{131} TO FREE BEEF INSULIN-I^{131} (B/F) AS A FUNCTION OF CONCENTRATION OF UNLABELED CRYSTALLINE BEEF INSULIN, CRYSTALLINE PORK INSULIN AND FISHER HUMAN INSULIN. In this and subsequent figures the insulin content of the Fisher crude powder was estimated to be 27.3 per cent by weight. Antiserum dilutions: GP 49 625, 1:6,000; GP 31 625 and 41 625, 1:4,000.

on this value. *2) "Cambridge pure human insulin,"* a lyophilized preparation of pure insulin,[1] was received from Dr. Ellis Samols, Royal Free Hospital, London, by courtesy of Prof. F. G. Young, Cambridge.

Insulin-I^{131} was prepared from crystalline beef or crystalline pork insulin as described previously (10). All insulin-antiserum mixtures in the same experiment were prepared with identical concentrations of I^{131}-labeled insulin and antiserum (added last in all cases) but varying concentrations of unlabeled insulin as indicated. After incubation for 3 to 5 days at 4° C, insulin-antiserum mixtures were applied to strips of Whatman 3 MM paper for chromato-electrophoresis as described previously (10, 13). After 45 to 90 minutes, antibody-bound insulin has moved about 3 inches from the site of application (origin) whereas unbound (free) insulin remains adsorbed to the paper at the origin. After drying, the paper strips were assayed for radioactivity in an automatic strip counter. Areas under the peaks of free and bound insulin-I^{131} were determined with a planimeter.

RESULTS

Antiserums from guinea pigs immunized with beef insulin were tested for their ability to discriminate between unlabeled beef insulin and Fisher human insulin competing against beef insulin-I^{131} (Figure 1). All data were obtained in a single experiment in which the same solutions of beef and human insulin were used for all antiserums. It is evident that beef insulin competed more strongly than human insulin in all antiserums and that the relative activity of beef and human insulin varied considerably among the three antiserums. The reaction of pork insulin was intermediate between that of human insulin and that of beef insulin. Depending on the antiserum tested and on the insulin concentration, it required from about 1.5 to 20 times as high a concentration of human insulin as beef insulin to reduce the ratio bound beef insulin-I^{131}/free beef insulin-I^{131} (B/F) to the same value. The results on guinea pig serum no. 49 625 were essentially the same (with respect to the relative activities of beef and human insulin) as those reported previously in another experiment with the same antiserum (10). A somewhat larger amount of beef insulin-I^{131} was used in the present experiment so that the initial ratio (in the absence of added unlabeled insulin) is slightly below that obtained previously.

[1] Purified on cellulose ion-exchange columns by the method of Dr. L. F. Smith, Cambridge.

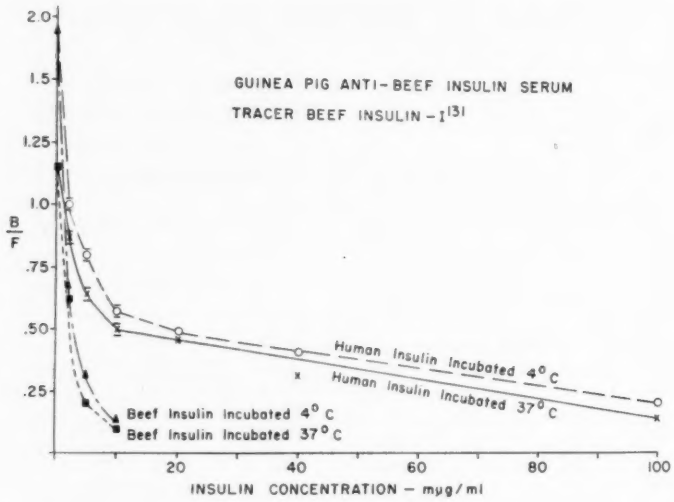
GUINEA PIG ANTI-BEEF INSULIN SERUM
TRACER BEEF INSULIN - I131
B/F
Human Insulin Incubated 4° C
Human Insulin Incubated 37° C
Beef Insulin Incubated 4° C
Beef Insulin Incubated 37° C
INSULIN CONCENTRATION - mμg/ml

FIG. 2. SAME AS FIGURE 1. GP antiserum 10 328, dilution 1:80.

To evaluate the influence of temperature on the difference in reactivities of beef and human insulin, another guinea pig antibeef insulin serum was studied in a separate experiment by incubation at 4° and 37° C (Figure 2). Although the B/F ratio at all concentrations was higher at 4° than at 37° C, as reported previously for human antiserums (14), there was no striking difference in the relative reactivities of the two insulins at the two temperatures.

Antiserums from human subjects treated with commercial mixtures of beef-pork insulin were studied in a similar manner (Figure 3). Again, all curves are from a single experiment in which

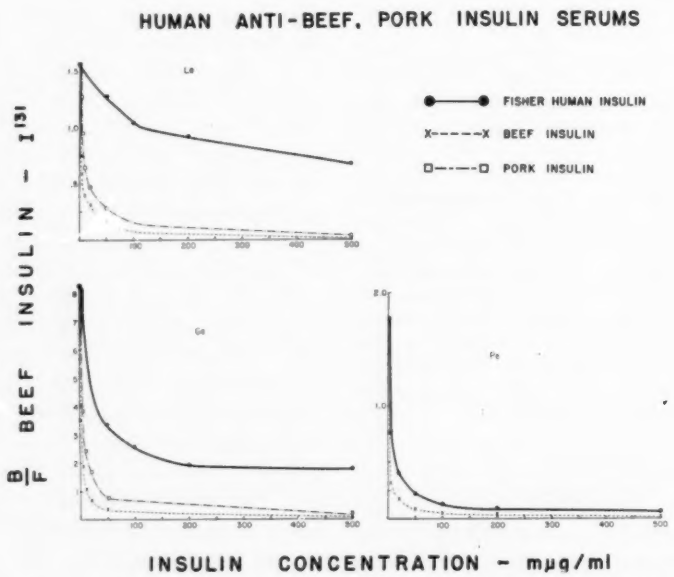

FIG. 3. SAME AS FIGURE 1. Antiserum dilutions: Le, 1:60; Ge, 1:400; Pe, 1:5.

the same insulin solutions were employed for all antiserums. It is evident that human antiserums may discriminate even more markedly than guinea pig antiserums between beef and Fisher human insulin. The relative reactivities of beef and human insulin, respectively, varied from about 3 to 200 depending on the antiserum tested and the insulin concentration employed. Pork insulin reacted somewhat more weakly than beef insulin but more strongly than human insulin in these antiserums.

In Figure 4 are shown results of similar studies in three guinea pig and three human antiserums in which the effects of beef insulin and the Cambridge *pure* human insulin are compared weight for weight in a single experiment. The same insulin solutions were employed for all antiserums. Results are similar to those of preceding experiments. In individual antiserums, the ratio between beef and human insulin concentrations required to reduce B/F to the same value varied over as much as a fivefold range at different B/F values.

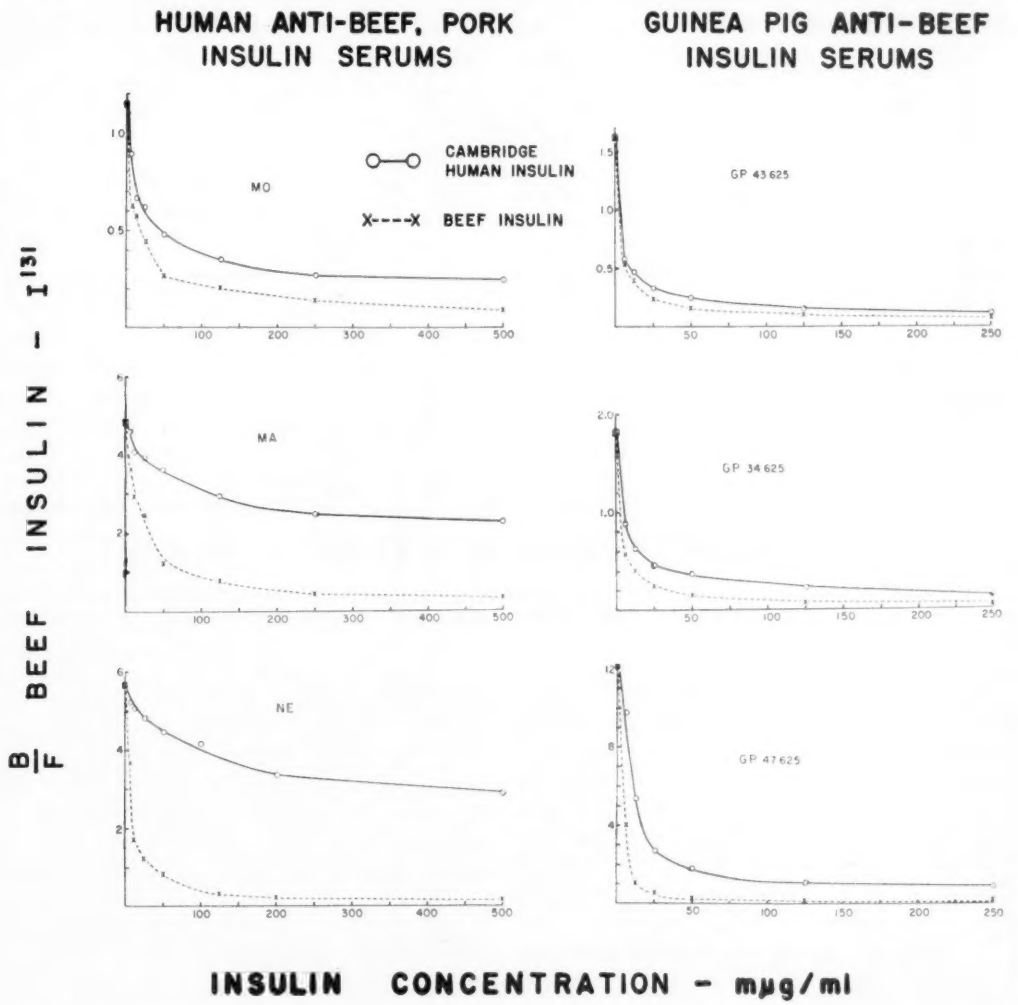

FIG. 4. B/F RATIOS FOR BEEF INSULIN-I^{131} AS A FUNCTION OF CONCENTRATION OF CAMBRIDGE HUMAN INSULIN AND CRYSTALLINE BEEF INSULIN. Antiserum dilutions: Mo and Ne, 1:20; Ma, 1:40; GP 43 625, 1:400; GP 34 625 and 47 625, 1:1,000.

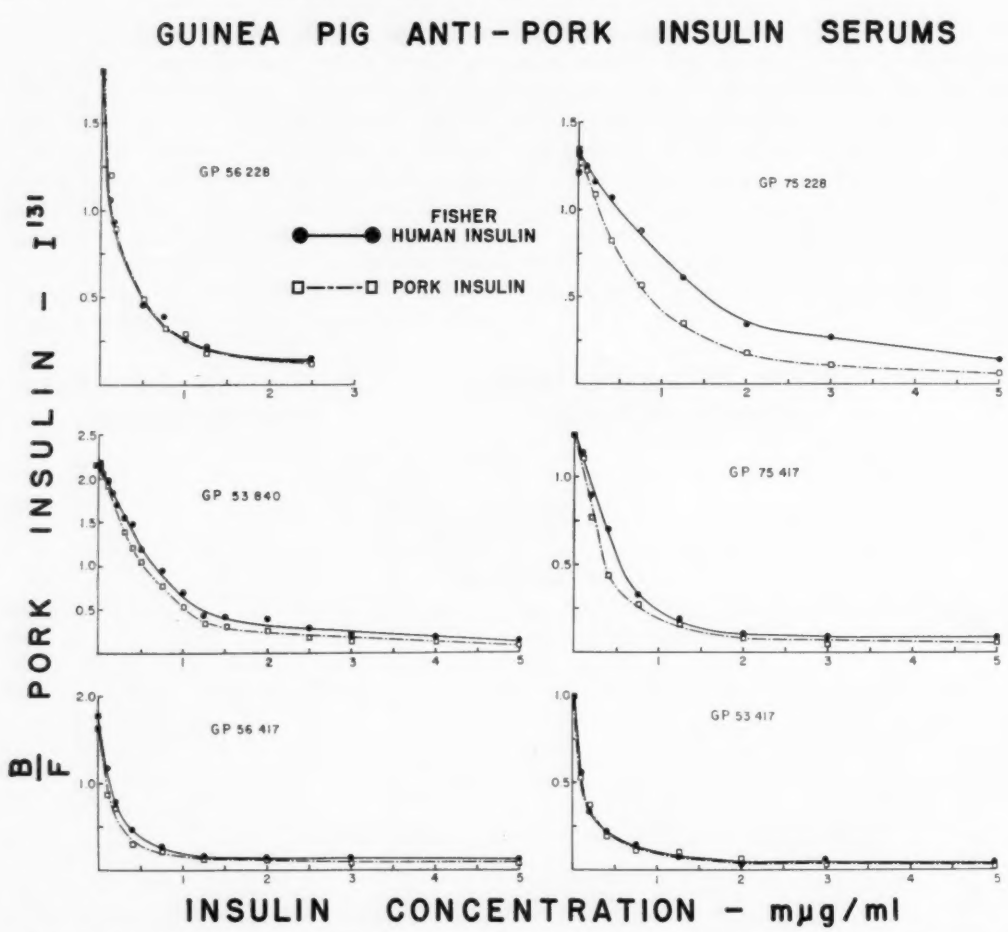

FIG. 5. B/F RATIOS FOR PORK INSULIN-I^{131} AS A FUNCTION OF CONCENTRATION OF FISHER HUMAN INSULIN AND CRYSTALLINE PORK INSULIN. Antiserum dilutions: 56 228, 1:2,000; 53 840, 1:8,000; 56 417, 1:10,000; 75 228, 1:1,500; 75 417, 1:10,000; 53 417, 1:60,000.

In contrast to results obtained with beef insulin-I^{131} in beef insulin-immunized guinea pigs, antiserums from guinea pigs immunized with pork insulin generally discriminated less readily between pork insulin and Fisher human insulin when tested against pork insulin-I^{131} (Figure 5). Two particular antiserums (GP no. 56 228 and GP no. 53 417) showed curves identical with pork insulin and Fisher human insulin.

In Figure 6 is presented a comparison of pork insulin and Cambridge pure human insulin, weight for weight, and Fisher human insulin estimated as 27.3 per cent insulin as calculated above, all competing against pork insulin-I^{131} in guinea pig anti-pork insulin serum no. 53 417, earlier (Figure 5) shown to exhibit identical behavior toward pork and Fisher human insulin. It is evident that the Fisher insulin, estimated at 27.3 per cent insulin by weight, the Cambridge pure insulin and the pork insulin all react identically and that this guinea pig antiserum does not distinguish among the two samples of human insulin and the sample of pork insulin. The results of the Fisher-Cambridge insulin comparison confirm results recently obtained by Dr. Ellis Samols, London, who found the Fisher crude powder "consistently equivalent to 25 to 30 per cent Cambridge insulin by weight" (15).

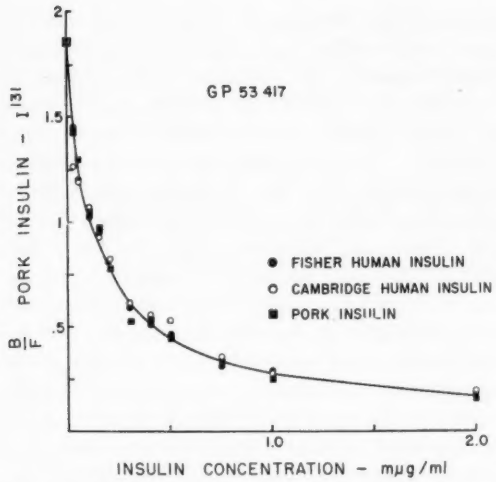

FIG. 6. B/F RATIOS FOR PORK INSULIN-I^{131} AS A FUNCTION OF CONCENTRATION OF FISHER HUMAN INSULIN, CAMBRIDGE HUMAN INSULIN AND CRYSTALLINE PORK INSULIN. Antiserum dilution, 1:30,000.

DISCUSSION

Recently, Grodsky and Forsham (16), employing a single beef-pork insulin antiserum from an insulin-resistant human subject as well as guinea pig antiserum to beef insulin, have tested various preparations of beef and human insulin (each at only two concentrations) for their ability to inhibit the binding of beef insulin-I^{131} to insulin antibodies. Their reported observations reveal that although a preparation of beef insulin assayed about 25 per cent *higher* in the human antiserum than in the guinea pig antiserum, all four preparations of human insulin assayed considerably *lower* in human antiserum than in guinea pig antiserum, using the same crystalline beef insulin as a reference standard for all assays. For one of the human insulin preparations, the assay in guinea pig antiserum was almost 300 per cent as high as in human antiserum, and for the other three preparations the human insulin assayed about 25 to 65 per cent higher in guinea pig than in human antiserum, as estimated from the published figure. Yet, it was concluded that "agreement between the immunoassay values for human insulin against beef insulin standards when either guinea pig or human antiserum was used was demonstrable." It is difficult to reconcile the authors' conclusion that human insulin and beef insulin react similarly in guinea pig and human insulin antiserums with the results of their studies on a single human antiserum which clearly dispute such a conclusion. "Agreement" of the order of magnitude reported hardly refutes our previous contention that beef insulin cannot be used indiscriminately as a standard for human insulin in immunologic systems (10). The more extensive data of the present study confirm previous observations that human insulin generally competes less strongly than beef insulin against the binding of beef I^{131}-insulin in antiserums from human subjects or from guinea pigs immunized with beef insulin and that the relative reactivities of human and beef insulin may vary widely among different antiserums. Furthermore, Burrows, Peters and Lowell (4) have reported on two human antiserums in which human insulin produced either no effect or only very slight effect on the binding of beef insulin-I^{131}. Earlier, Lowell (2) had reported that administration of human insulin to an insulin-resistant patient was effective in lowering blood sugar although beef insulin in the same dosage was completely ineffective, and Lerman (17) had emphasized that insulin antibodies in insulin-resistant human subjects are weakest when tested against human insulin. More recently we have directly compared human insulin binding (employing human insulin-I^{131} with unlabeled human insulin) and beef insulin binding (employing beef insulin-I^{131} with unlabeled beef insulin) in eight human antiserums (18). Weaker binding of human insulin than of beef insulin was observed in almost all cases. It is only to be expected that the cross reaction of unlabeled human insulin with I^{131}-labeled beef insulin would be still weaker. The evidence of previous studies and that of the experiments reported here clearly refute the notion that beef and human insulin react similarly with human antibeef-pork insulin serums or with guinea pig antibeef insulin serums.

Grodsky and Forsham (16) also studied the Fisher preparation of human insulin, which they report as having been recently assayed in the Lilly Research Laboratories at 4.08 U per mg.[2] Applying this new assay to a figure previously published by the present authors, Grodsky and Forsham concluded *1*) that the beef insulin and human insulin curves could be superimposed and

[2] Based on a reference standard of 27 to 29 U per mg.

therefore that these two insulins reacted identically against beef insulin-I^{131} in guinea pig anti-beef insulin antiserums, and *2*) that the proportionality between beef and human insulin in the *original* data "varied only from 0.45 to 0.55 regardless of the point chosen along the curves for the calculations." However, as is evident from the reproduced figure in their paper as well as from the original data, this statement is inaccurate, since the proportionality factor ranged from about 0.3 to 0.6. It might also be noted that the original figure showed values for the Tietze insulin falling along the same curve as the Fisher insulin, but the former were omitted from the reproduced figure. A repeat experiment with the same antiserum (GP no. 49 625), presented in the current study (Figure 3), completely confirms the previously reported results. Thus, the same human insulin solution, if assayed at different dilutions with reference to the beef insulin curve, would have yielded estimates differing by almost 100 per cent in the antiserum reported. The results of the present study confirm and extend previous observations that the relative reactivities of human and beef insulin may vary over a range considerably greater than 100 per cent and even as great as 500 per cent at different concentrations in the same antiserum. It is evident from Figures 1–4 that no single correction factor for the Fisher human insulin concentration could possibly superimpose the curves for human and beef insulin in all antiserums.

If the Fisher insulin were significantly lower in insulin content than previously estimated, the findings of the present study would require the interpretation that human insulin reacts more strongly than pork insulin with guinea pig antibodies to pork insulin in competing with pork insulin-I^{131}. This is a *most unlikely* result. It is quite possible that there has been a greater loss of *hormonal* potency in the Fisher insulin than previously estimated,[3] particularly since a longer period of time has elapsed. Nevertheless, the entire insulin molecule certainly did not disappear from the powder and that part of its structure responsible for immunologic activity could remain essentially intact even if hormonal activity were abolished. Desoctapeptide beef insulin (lacking 8 amino acids from the C-terminal end of the B chain) is completely inactive hormonally (19) and yet reacts in the immunologic system (20). Therefore, loss of hormonal potency, if it has occurred, cannot necessarily be considered equivalent to a diminution in immunologic potency. By immunoassay, the Fisher insulin can certainly not be considered significantly weaker than originally estimated by us, if consideration is given to the comparisons with the Cambridge pure insulin and the crystalline pork insulin. The identity of reaction of Fisher insulin (on the basis of its previously assigned value) and Cambridge insulin (15, and present study), and the identity of reaction of Fisher human insulin and pork insulin in certain guinea pig antipork insulin serums indicate that the immunologically active insulin content of the Fisher preparation has remained essentially unchanged as estimated on the basis of its original assay of hormonal activity. It should be appreciated that unless the water content of dried insulin powders is determined precisely, an uncertainty of about 10 per cent must always exist regardless of the method of assay.

On the basis of the amino acid sequences of the various mammalian insulins (11, 12), it might be anticipated that human insulin would show a closer immunologic relationship to pork insulin than to other animal insulins which differ from human insulin at two sites. Previous studies had suggested that the 8 to 10 sequence of the insulin A chain is an important site of reaction with antibodies in the serums of human subjects immunized with beef-pork insulin mixtures (5). Even if pork insulin itself were not antigenic, the reaction of pork insulin at this site could be interpreted in the same light as the reaction of human insulin (5), namely, that antibody once formed in response to sensitization with an insulin distinctly foreign in structure at a small site can then react with other insulins differing slightly at this site.

[3] This loss, if it does occur, need not be the same in all hands. Our supplies have been kept in a desiccator at 4°C since receipt in July 1959 and no diminution in immunologic reactivity has been observed to date. Among other factors, simple deliquescence can account for a significant increase in weight, thereby decreasing the potency (hormonal or immunologic) per unit weight. Furthermore, crystalline beef insulin (lot no. 535664), received by us in 1955, has also been used by many other workers who have relied on the original assay.

However, aside from differences in amino acid sequences, the various insulins might well exhibit differences in secondary or tertiary structure as well, a suggestion supported by distinct immunochemical differences between sperm whale and pork insulin (21), which are reported as identical in amino acid sequences (11, 22). Since the structure of guinea pig insulin has not yet been elucidated, it is not possible to speculate on the potentially antigenic sites of beef and pork insulin in this species. The very close similarity (or identity) of reaction of pork and human insulin with guinea pig antibodies against pork insulin suggests that the C-terminal end of the B chain might not be antigenic. That there is at least one other antigenic site is deducible from observations that desoctapeptide beef insulin reacts with guinea pig antipork insulin serums (23). However, an additional contribution of the C-terminal residue of the B chain to antigenicity of pork insulin can certainly not be excluded. Furthermore, the similarity of pork and human insulin does not extend to the reaction against beef insulin-I^{131} (or even against pork insulin-I^{131}) in beef insulin-immunized guinea pigs or humans (5). Since beef and pork insulin contain the same C-terminal amino acid in the B chain, no *differences* in antigenicity of this group can be expected and we must again postulate structural differences beyond those of specific amino acid sequence to account for all findings. In any event, these theoretical speculations are outside the practical purpose of the present study, namely, the procurement of antiserums suitable for immunoassay of endogenous plasma insulin in man under conditions that dispense with the need for human insulin.

SUMMARY AND CONCLUSIONS

1. Previous observations of variable and occasionally marked differences in reaction of beef insulin and human insulin competing with beef insulin-I^{131} in human antibeef-pork insulin serums and in guinea pig antibeef insulin serums are confirmed and extended.

2. Antiserums from guinea pigs immunized with pork insulin exhibit close similarity of reaction with pork and human insulin. In two antiserums, pork and human insulin reacted identically when competing against the antibody-binding of pork insulin-I^{131}. Employment of these antiserums for the immunoassay of endogenous plasma insulin in man obviates the necessity for employment of human insulin as a standard.

3. Human insulin (Fisher), previously estimated at 27.3 per cent insulin by weight, was assayed against pure human insulin (Cambridge) as standard. Present results confirm the accuracy of the previous estimate for immunologically active insulin content of the Fisher preparation.

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THE FREQUENCY IN JAPAN OF CARRIERS OF THE RARE "RECESSIVE" GENE CAUSING ACATALASEMIA

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Although it is increasingly recognized that many rare recessive genes have effects in the heterozygous state which can be detected by suitable laboratory tests, with the implication that such genes may make a significant contribution to subclinical departures from health, there is a real paucity of knowledge concerning the actual distribution in populations of such heterozygotes. Until now it has not been possible to study the distribution of heterozygotes for a rare "recessive" gene with a simple, quick, and accurate method suitable for survey purposes. It will be the object of this paper to describe such a method for detecting the heterozygous carriers of a particular rare "recessive" gene. Survey data will be presented indicating significant local variations in gene frequency. The implications of such variations for certain basic genetic problems will be briefly explored.

The trait in question is acatalasemia, a recessively inherited condition characterized by the absence of detectable tissue catalase activity (1). Heterozygotes for this gene have been shown to exhibit approximately half-normal values of catalase activity (2). In a previous paper, it was noted that the application of standard genetic formulae led to an estimate of 0.003 for q, the frequency of the gene responsible for the trait, from which the frequency of homozygotes was calculated to be 0.00002, and of heterozygotes, 0.006; i.e., 2 and 600 per 100,000 Japanese, respectively (3). The many assumptions which went into these calculations were discussed briefly, with the statement that in general the computations were of such a nature as to result in inflated estimates of the gene frequency, and it was pointed out that the true gene frequency might well be of the order of 0.0015. It is now possible to pursue this point further.

METHODS OF STUDY

A. Composition of the population sample

Screening for hypocatalasemia was performed on blood samples received in the Clinical Laboratories of the Atomic Bomb Casualty Commission (ABCC) in Hiroshima and Nagasaki. Bloods were derived from three sources: *1*) radiation-exposed and nonexposed individuals, who are voluntarily participating in the ABCC Adult Health Study [1] (4); *2*) children seen in the Child Health Study at ABCC (5); and *3*) "referrals" of blood specimens to the ABCC by local practitioners, where blood only was received with a request for laboratory determinations. The third group constituted less than 5 per cent of the total number of specimens examined; as far as is known, catalase abnormalities never formed the basis for referral. Between 30 and 60 samples were screened daily in Hiroshima and approximately one-third that number in Nagasaki over a period of about 15 months. No attempt was made at a rigorous exclusion of related individuals, but from the method of selection, it seems unlikely that related individuals (parent-offspring or sibling pairs) constituted more than 15 per cent of the sample. Blood specimens that were positive to the screening test were subjected to the more precise assay for erythrocyte catalase activity, as described below.

B. Assay methods

1. Screening method. Two ml of blood was transferred to 20 ml of tap water, with care taken to wipe the outside of the pipet and flush it with the diluent. After gentle mixing, 1 ml of this solution was *immediately* pipetted into 5.0 ml of 0.01 N hydrogen peroxide in 0.01 M phosphate buffer, pH 6.8. After exactly 60 seconds, 2 ml of 2 N sulfuric acid was added, followed by 7 ml of

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[1] Since it has been found that there is no difference between erythrocyte catalase activity among exposed and nonexposed individuals, possible radiation effects need not be considered (3).

0.005 M potassium permanganate. The latter solution may be added to a row of tubes at any convenient time after addition of the acid. If a pink color persists after addition of the permanganate, catalase is present; if the solution remains colorless, catalase is either absent or in low concentration, provided the hemoglobin is within normal limits.

This test is based on two premises: *1)* That a given amount of catalase, in a given time interval, decomposes a fixed amount of hydrogen peroxide, the amount remaining in effect being measured by titration with permanganate. The concentration of permanganate chosen was such that only individuals with catalase activity less than 3 standard deviations below the mean catalase activity of a control population would show a positive reaction. *2)* This in turn is based on the assumption that the hemoglobin in the blood sample is within normal limits, since it has been shown that there is a linear relationship between erythrocyte catalase activity and hemoglobin concentration (3).

Thus, false positive results are to be expected in those instances where the hemoglobin concentration is low. Because this is primarily a screening test, adjusting the hemoglobin concentration has of necessity been omitted, but the test has been designed so that in the majority of instances only blood samples with a hemoglobin of less than 7.5 g per cent will give a false positive result. However, since hypocatalasemia is not necessarily ruled out in the presence of anemia, a precise assay for catalase activity must be performed whenever the screening test is positive.

In this study it was customary to withdraw the 2-ml sample from a tube of heparinized or citrated venous blood. However, the method is equally applicable to fresh blood, drawn from a finger or earlobe prick into a hemoglobin pipet and immediately diluted with tap water as described. Further, if it is inconvenient to carry all the reagents into the field, a heparinized pipet may be used, and the blood transported to the laboratory for the screening test. However, care should be taken to avoid unduly long intervals between obtaining the blood and screening, unless the sample is refrigerated. Further, the latter method suffers from the disadvantage that in the case of positive results, where the precise assay becomes necessary, a second sample of blood must be obtained at a later time.

2. Assay for erythrocyte catalase activity. The method for determining erythrocyte catalase activity (K_{cat} activity) has already been described (3). A preliminary attempt was made to determine the frequency with which false positive (positive screen with normal K_{cat}) and false negative results (negative screen with low K_{cat}) occurred. In a series of 120 "blind" tests, in which both the screening and K_{cat} assay were performed on individual samples, there were no instances of false negative results, but false positive results did occur, either because of a low hemoglobin level, as discussed above, or a combination of a K_{cat} value at the lower limit of normal and a somewhat lowered hemoglobin level. In the total survey series, about 1 per cent of the screening tests gave false positive results. It would thus appear that in surveying a large population, false positive results can be minimized by determination of the hemoglobin values *and* performance of the K_{cat} assay, whereas false negative results (e.g., missed hypocatalasemics) apparently occur rarely, if at all.

TABLE I

The frequency of hypocatalasemia in six different surveys

Population	Survey	No. examined	No. hypocatalasemic	Frequency of hypocatalasemia
Hiroshima Japanese	Present study	10,679	10*	0.000936
Nagasaki Japanese	Present study	2,968	1	0.000337
Okayama Japanese	Reference 7	1,500	0	0.000000
Nagano Japanese	Reference 7	1,975	30	0.015190
Shimane Japanese	Reference 7	618	6	0.009709
Okayama Koreans	Reference 7	775	7†	0.009032

* Includes one mother-daughter pair.
† Includes one pair of siblings.

C. Family studies

In all instances in which a positive screening test followed by a K_{cat} assay revealed the presence of a hypocatalasemic individual, a second sample of fresh blood was obtained at a later date and K_{cat} activity redetermined. In every case, the second result confirmed the first. At this second visit, a careful pedigree was obtained from the proband; subsequently, blood samples were obtained from as many other members of the family as possible. In this way, individuals from 10 families were studied in detail with respect to K_{cat} values.

RESULTS

Table I summarizes the survey findings in Hiroshima and Nagasaki. The probability of as great or a greater difference between the two cities if the expectation for both is the same, as evaluated by the exact method for 2×2 tables (6), is 0.28, clearly nonsignificant. Figure 1 and Table II present the data obtained in studies on the kindreds of the 11 hypocatalasemic subjects encountered in the course of the survey. The column labeled "pedigree position" refers to the position of each individual in the pedigrees of the 10 kindreds concerned, as shown in Figure 1.[2]

[2] Although 11 hypocatalasemics were encountered, 2 were related as mother and daughter, so that only 10 kindreds were represented.

PEDIGREES OF FAMILIES WITH CARRIERS (HYPOCATALASEMICS) FOR ACATALASEMIA

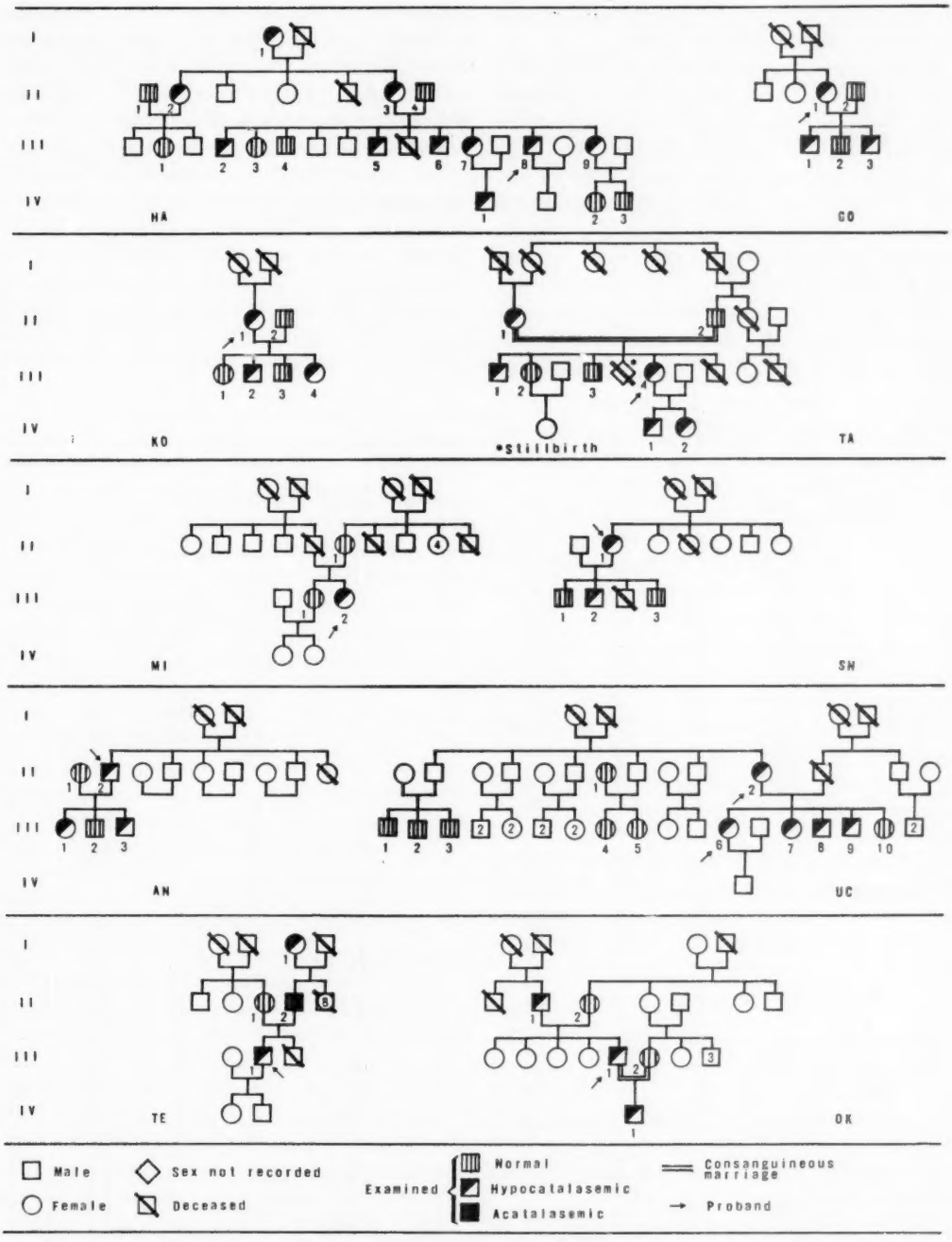

FIG. 1. THE RESULTS OF FAMILY STUDIES ON INDIVIDUALS FOUND TO HAVE HYPOCATALASEMIA IN THE COURSE OF THIS SURVEY.

In every kindred studied except one, other members of the kindred were found to be carriers; in this exceptional kindred, only a single parent and one sibling were available for study.

Means, ranges, and standard deviations of K_{cat} values for normal and hypocatalasemic subjects in this study are compared in Table III with those previously reported (3). In the previous study, a significant difference was noted between the mean for normal control determinations performed on subjects being studied at the ABCC and the mean for presumed normal subjects in families with acatalasemic members. This was attributed to the fact that it was often necessary to store the specimens from the latter group for several days before it was feasible to perform catalase determinations, presumably with some loss of catalase activity. In the present study, in which it was possible to perform the determinations almost immediately after the blood was drawn, the normal values correspond very well with ABCC clinic control values, thus corroborating the suggestion of loss of catalase activity with storage.

The present data confirm in all respects the previously advanced hypothesis that hypocatalasemia and acatalasemia are related as heterozygote and homozygote and, further, demonstrate that the present screening technique is highly efficient in the recognition of hypocatalasemia. Japanese investigators are now conducting surveys at widely scattered points in Japan. The most advanced of these surveys are those of Takahara (7), some of

TABLE II

K_{cat} values for members of kindreds in which hypocatalasemia occurs

Family	Pedigree position	Sex	Age	K_{cat}	Family	Pedigree position	Sex	Age	K_{cat}
	I-1	F	76	2.64		II-1	F	42	2.43*
	II-1	M	54	5.75		II-2	M	45	5.55
	II-2	F	50	2.32	GO	III-1	M	10	2.54
	II-3	F	53	2.22		III-2	M	16	5.15
	II-4	M	55	5.10		III-3	M	19	2.52
	III-1	F	22	5.26					
	III-2	M	12	2.44		II-1	F	61	2.71
HA	III-3	F	14	5.55		II-2	M	66	3.92
	III-4	M	15	4.47		III-1	M	25	1.94
	III-5	M	21	2.49	TA	III-2	F	26	5.79
	III-6	M	25	2.08		III-3	M	29	5.40
	III-7	F	27	2.53		III-4	F	38	2.47*
	III-8	M	28	2.12*		IV-1	M	5	2.54
	III-9	F	29	2.23		IV-2	F	8	2.47
	IV-1	M	2	2.36					
	IV-2	F	3	4.40		II-1	F	51	2.23*
	IV-3	M	5	5.55		III-1	M	16	5.11
					SH	III-2	M	18	2.95
	II-1	F	44	2.14*		III-3	M	23	5.36
	II-2	M	49	6.28					
KO	III-1	F	7	5.66					
	III-2	M	10	2.92		II-1	F	38	5.30
	III-3	M	12	5.71		II-2	F	46	2.73*
	III-4	F	20	2.93		III-1	M	22	4.88
						III-2	M	19	5.11
	II-1	F	67	5.30		III-3	M	12	5.16
MI	III-1	F	40	5.11	UC†	III-4	F	17	5.15
	III-2	F	25	2.35*		III-5	F	14	6.33
						III-6	F	26	2.66*
	II-1	F	32	5.05		III-7	F	22	2.72
	II-2	M	41	2.35*		III-8	M	21	2.57
AN	III-1	F	11	2.51		III-9	M	19	2.98
	III-2	M	10	6.25		III-10	F	16	4.80
	III-3	M	8	2.77					
						II-1	M	63	2.21
	I-1	F	80	2.83		II-2	F	59	5.07
TE	II-1	F	56	5.73	OK	III-1	M	35	2.37*
	II-2	M	60	0		III-2	F	30	5.33
	III-1	M	33	2.67*		IV-1	M	8	2.96

* Proband.

† The two probands, II-2 and III-6, of kindred UC were ascertained independently of each other.

TABLE III

A comparison of K_{cat} values in control individuals and in members of kindreds segregating for hypocatalasemia and acatalasemia

Source and type of patient	No.	K_{cat} values Mean	SD	Range
ABCC Clinic control	259	5.38	0.73	3.90 – 7.47
Non-acatalasemic members of kindreds segregating for acatalasemia (Reference 3):				
Normal	55	4.97	0.61	3.96 – 6.47
Hypocatalasemic	31	2.17	0.35	1.48 – 2.87
Kindreds found to be segregating for hypocatalasemia (present study):				
Normal	31	5.30	0.53	3.92 – 6.28
Hypocatalasemic	37	2.51	0.27	1.94 – 2.98

the results of which have been reproduced in Table I. He has studied three areas, namely, Okayama, a city of some 236,000 individuals situated on the island of Honshu, facing the Inland Sea, and two semi-mountainous regions of central and western Honshu, located in Nagano and Shimane prefectures, respectively, where several sibships with acatalasemic members have been encountered. The figures for Okayama Japanese (college students, 740; patients attending an otology clinic, 419; and randomly selected adults, 341) are in satisfactory agreement with those for Hiroshima and Nagasaki. The figures for Nagano and Shimane are based on surveys of primary school children from the first through the sixth grades. The sample undoubtedly includes some sibling pairs. Furthermore, consanguinity rates are relatively high in Nagano; Kubota and others (8) reported total values for different localities ranging from 9 to 28 per cent. Although consanguinity rates for Shimane have not been established, on the basis of reports for other widely separated rural areas in Japan (Summary in Reference 9), it is probably safe to assume that the frequency of consanguineous marriages corresponds to that of other similar sections of the country. Thus, it seems quite likely that there is a significantly greater proportion of related individuals in these two samples than in those for the three cities studied thus far. These facts raise some theoretical difficulties in the interpretation of the usual tests for the significance of frequency differences, since a single extended kindred might be contributing most of the affected individuals in Nagano or Shimane. The probability of observing a difference equal to or exceeding that between the combined observations in Hiroshima and Nagasaki and those in Nagano and Shimane (if the expectation for the areas concerned were the same and all observations independent) as computed by any one of several methods, is very low—well beyond the usual significance levels. While allowance for the possible biological relatedness of some of the individuals in the sample would reduce to some degree the apparent significance of the differences, it could scarcely eliminate them. In round terms, the frequency of carriers in the Nagano and Shimane areas is some 10 to 15 times that in Hiroshima and Nagasaki.

The preliminary findings in persons of Korean descent residing in Okayama, also summarized in Table I, are especially interesting. This group, composed of schoolboys from four different districts in Okayama, was surveyed after acatalasemia was recently encountered in three Korean siblings in Tokyo (10). In a previous publication (3) it was argued, from the widespread distribution throughout Japan of the relatively few sibships with acatalasemic members, that the responsible gene was one of considerable antiquity. The demonstration of the gene in Koreans can be regarded as reinforcement for that suggestion, since the principal route by which the Japanese reached their islands appears to have been by way of Korea (11–15), and it seems more likely, in view of the history of Japanese-Korean relationships, that the gene was introduced into Japan by way of Korea than that recent Japanese-Korean contacts have resulted in the introduction of the gene into Korea. On the other hand, the possibility of multiple independent origins of the gene, in both Korea and Japan, cannot be completely disregarded.

DISCUSSION

Clearly there are significant local variations in Japan in the frequency of heterozygotes for the acatalasemia gene. The concept suggested by these data, of "pockets" wherein certain rare and "pathological" recessive genes are represented by unusually high frequencies, is of course not new (16), but previously the means of directly verifying and quantitating this concept have not been at hand. This demonstration has important implications for the use of certain genetic formulae,

which are discussed below. In a previous publication, the frequency in Japan of carriers of the gene for acatalasemia was estimated at 0.006 (3). This estimate was based on a formula derived by Dahlberg (17), namely,

$$q = \frac{c(1 - k)}{16k - 15c - ck} \qquad [1]$$

with the frequency of heterozygotes then calculated from the relationship,

$$\text{heterozygotes} = 2q(1 - q)(1 - \alpha) \qquad [2]$$

where q = the frequency of the recessive gene responsible for acatalasemia; c = the proportion of first-cousin marriages among the population as a whole, assumed from previous studies in Japan to be 0.06 (18, 19); k = the proportion of first-cousin marriages among the parents of homozygous individuals, assumed from the data on 17 sibships to be 0.59 (3); and α = the coefficient of inbreeding for the population, assumed from previous studies to be 0.004 (18).

Kimura (20) has now provided a more general, maximum likelihood-type formula, which has the advantage of utilizing for the estimate all the data available concerning consanguinity in the parents of affected individuals. Kimura has also derived the formula for the variance of the estimate, which was not available for Dahlberg's approach. With this method, substituting values to be found in our previous paper, the gene frequency is estimated at 0.00082, with a standard error of 0.0053. Thus, within the range of 1 standard error above and below the estimated gene frequency are to be found values from zero to 0.0061, the latter corresponding to a carrier frequency of 0.0121. The estimate has so large an error as to encompass the value obtained with the Dahlberg equation and also both the observed Nagasaki-Hiroshima and the Nagano-Shimane values, even though these latter two values differ significantly from each other. Although, with only 17 sibships on which to base the gene frequency estimate, a large error was to be expected, the actual magnitude of this error is sobering. It is clear that many more data must be collected before it will be possible to test with any precision the degree to which the gene frequencies predicted for various regions in Japan and the total area will depart from the frequencies which can be determined by suitable surveys. At a first approximation, it would appear that at least 100 additional sibships in which acatalasemia is segregating will be necessary to refine the gene frequency estimate to the point where comparisons between observation and expectation become meaningful.

The importance of estimating the frequency of the carriers of a variety of recessive genes is such that a considerable expenditure of effort in pursuing this favorable test situation would be warranted if it promised to yield a critical evaluation of the usefulness of Dahlberg's and Kimura's formulae. However, there is now reason to suspect that human populations depart from the mathematical models on which the formulae are based to an extent which renders a close correspondence between prediction and observation unlikely. The formulae are based on certain rather sweeping assumptions. Although geneticists have recognized for some years that these assumptions involve oversimplifications of highly complex situations (Discussion in 3, 17, 18, 21, 22), the use of the formulae was nevertheless justified because, in the case of rare recessive traits, it resulted in an approximation to a figure usually not otherwise available. These assumptions state that:

1) *There is a uniform gene frequency* throughout the total area under consideration, an assumption whose incorrectness in the case of acatalasemia has already been demonstrated by graphic evidence.

2) *The population is in genetic equilibrium;* i.e., the balance between mutation, selection, and population structure is such that the gene frequency has stabilized.

3) *The population constitutes one interbreeding unit.* Patently, two individuals, one from Hokkaido and one from Kyushu, have much less opportunity of marrying than a pair from Honshu, but migration between the hierarchy of "isolates" (or neighborhoods) within "isolates" which comprise a population is assumed to occur at a rate sufficient to result in a satisfactory approximation to the model.

4) *The disease is genetically homogeneous;* i.e., all affected individuals result from mutation at the same locus, again an assumption that seems untrue for acatalasemia (3 and unpublished observations).

It is clear that we are not yet in a position to judge just how far human populations depart from assumptions 2, 3, and 4, or the manner in which these departures influence mathematical prediction. There are indications, however, that the departures may be very considerable. On the other hand, the data of this paper, plus the findings of Takahara, do give us rather graphic insight into the departures which may exist from assumption 1. It is clear that these departures may be of such magnitude as to have important implications for the use of these specific formulae and for the manner in which data on consanguinity effects are utilized for a variety of genetic calculations. Out of a multitude of possible illustrative models, two simple alternatives suggested by the present survey data may be considered. These alternatives are certainly abstractions in that, while they represent a step toward biological realities, they are still gross oversimplifications. Without these oversimplifications, however, the principal point could be buried in a mass of mathematical detail. Let us assume that in a group of 10,000 individuals there occurs a completely neutral and completely recessive gene. This gene is limited to three extended kindreds, in each of which 10 (heterozygous) members possess it. This group moves into a new and favorable geographical area. Circumstances are such that this group immediately becomes subdivided into three subgroups ("neighborhoods") comprising, respectively, 10, 20, and 70 per cent of the population, with differing rates of inbreeding and with no exchange of members. It may be assumed that at the time such a group first moves into an area and distributes itself to occupy the desirable portions of the new territory, biologically related individuals (kindreds) will tend to remain together. We will postulate that in the smallest group, 10 per cent of all marriages involve first cousins ($c = 0.10$), while in the intermediate group the frequency of first-cousin marriage is 5 per cent, and in the largest group, 1 per cent. In time, all subgroups undergo a 100-fold increase in numbers, so that the total population becomes 1,000,000, with gene carriers increasing proportionately. No further mutations resulting in this particular gene occur, and there is assumed to be no genetic drift.

Let us now explore the consequences for the application of Dahlberg's formula of the location of the three kindreds carrying the hypothetical gene. We use Dahlberg's rather than Kimura's formula only because of the mathematical simplicity of the former. In model 1, it is assumed that one of these kindreds finds a place in each of the three "neighborhoods." In model 2, it is assumed that all three kindreds come to reside in the largest "neighborhood." The differences which these accidents of distribution can introduce into estimates of q as calculated from the over-all average of c and k are shown in Table IV. For each of the subpopulations, k has been derived by substitution of the given values of q and c into Formula 1. Then, as would be the case in most attempts to calculate gene frequencies, an average value of c and k for the entire population has been obtained. It is apparent that the estimates of q for the total population resulting from substitution of the *average* value of c and k in Formula 1 differ for the two models by a factor of 5.

Although this particular example is undoubtedly somewhat extreme in the disparate degree of inbreeding in the subpopulations, as well as in the rarity of the gene concerned, and in the simplifying assumptions which are made regarding the characteristics of the population, it is clear that lesser disparities, such as can exist within even a single Japanese city (19), can easily result in

TABLE IV

Comparison of calculations for the frequency, q, of a rare recessive gene in two contrasting population models (described in the text)

Proportion of population	q	c	k
%			
Model 1			
10	0.0050	0.10	0.60
20	0.0025	0.05	0.58
70	0.000714	0.01	0.48
	$q_{av} = 0.0015$	$c_{av} = 0.027$	$k_{av} = 0.59$
	$q_{calc} = 0.0012$		
Model 2			
10	0.0	0.10	
20	0.0	0.05	
70	0.00214	0.01	0.24
	$q_{av} = 0.0015$	$c_{av} = 0.027$	$k_{av} = 0.24$
	$q_{calc} = 0.0060$		

TABLE V

A comparison of the consequences of first-cousin marriage in the two different population models described in the text *

Proportion of population	First-cousin marriages						Marriages of unrelated persons			
	Total no. (n)	qF	qFn	$q^2(1-F)$	$q^2(1-F)n$	Total affected	Total no. (n')	q^2	q^2n'	Total
%										
Model 1										
10	100,000 × 0.1 = 10,000	(0.005)(0.06) = 0.00030	3	$(0.005)^2(0.94)$ = 0.000024	0.24	3.24	90,000	0.000025	2.25	5.49
20	200,000 × 0.05 = 10,000	(0.0025)(0.06) = 0.00015	1.5	$(0.0025)^2(0.94)$ = 0.00000588	0.06	1.56	190,000	0.00000625	1.18	2.74
70	700,000 × 0.01 = 7,000	(0.000714)(0.06) = 0.0000428	0.300	(0.000714)(0.94) = 0.000000479	0.00335	0.303	693,000	0.000000510	0.353	0.656
Total	27,000					5.10	973,000		3.78	
	Incidence, first cousins $= \frac{5.10}{27{,}000} = 0.00018889$			Incidence, unrelated $= \frac{3.78}{973{,}000} = 0.00000388$			Ratio $= \frac{0.00018889}{0.00000388} = 48.68$			
Model 2										
10	10,000	0	0	0	0	0	90,000	0	0	0
20	10,000	0	0	0	0	0	190,000	0	0	0
70	7,000	(0.00215)(0.06) − 0.000129	0.9	$(0.00215)^2(0.94)$ −0.00000434	0.03	0.93	693,000	0.0000046225	3.2	4.13
Total	27,000					0.93	973,000		3.2	
	Incidence, first cousins $= \frac{0.93}{27{,}000} = 0.00003444$			Incidence, unrelated $= \frac{3.2}{973{,}000} = 0.00000329$			Ratio $= \frac{0.00003444}{0.00000329} = 10.47$			

* Symbols have the following meaning: q = gene frequency; F = coefficient of inbreeding for first-cousin marriages, namely, 0.06; and n and n' refer to size of subpopulations. The frequency of homozygotes resulting from the marriage of first cousins is $qF + q^2(1 - F)$ (i.e., columns 3 + 5), while the frequency from the marriage of unrelated persons is simply q^2 (column 9).

calculations differing by a factor of 2 or 3. It should be re-emphasized that these are vastly oversimplified models, chosen from a multitude of possibilities, but a variety of other models would serve to make the same point of the influence of population structure on gene frequency estimates. Furthermore, only one of several ways in which populations may depart from the model has been considered here. Other possible, disturbing influences include such factors as fertility differentials, recent relaxation of inbreeding, and assortative mating. Fortunately, the increasing ability to detect a variety of heterozygous carrier states promises to substitute observation for the predictions of oversimplified statistical models, and at the same time may provide the insight to improve the models.

These simple models also have some interesting implications for attempts to utilize inbreeding effects for a variety of computations (23). Returning to the situation described above, assume that the trait associated with this hypothetical recessive gene is readily identifiable, so that it would be scored in studies on inbreeding effects. Further, assume only two types of marriages in the two populations, namely, between first cousins or between unrelated individuals, and, for this generation, stability in population numbers. The frequency of appearance of affected individuals from the two types of marriages in consequence of the two different models of population structure has been computed in Table V. In the first case, the ratio of proportion affected from first-cousin marriages to proportion affected from non-consanguineous marriages is approximately 49, whereas in the second case, it is approximately 10. Stated in terms of the ratio of the "inbred load" to the "randomly mating load" of Morton, Crow and Muller (23), these correspond to ratios of 763 and 151, respectively.

The significance of a knowledge of population structure for attempts to calculate "mutation" versus "segregation" loads is thus obvious, but will not be discussed in detail at this time. However, it may be noted that this factor has been inadequately dealt with in some current treatments of the subject (23, 24). It should also be apparent from the model that conclusions regarding the number of loci at which recessive genes may occur with a given phenotypic effect (e.g., deaf mutism), which are based upon an apparent discrepancy between the observed frequency of the phenotype and the frequency of consanguinity among the parents of affected individuals, may also be biased by this "neighborhood effect." The bias can be of such a degree that, for the present, attempts on the basis of consanguinity data to estimate the number of recessive genes capable of producing a given phenotype, and the deductions drawn therefrom (25), cannot be taken very seriously, a fact which if not already apparent on theoretical grounds would be suggested by the wide discrepancy between calculations based on the data of Stevenson and Cheeseman (26) from Northern Ireland and Lindenov (27) from Denmark.

SUMMARY

A rapid and simple method for detection of hypocatalasemic heterozygotes for the gene responsible for acatalasemia is described. A total of 13,647 blood specimens from individuals residing in Hiroshima and Nagasaki has been examined in this manner, and the frequency of hypocatalasemics was found to be 0.09 per cent. Surveys in other parts of Japan have revealed frequencies of the order of 1.4 per cent. This unevenness in the distribution of a rare "recessive" gene, taken in conjunction with the known variation from locality to locality in the frequency of inbreeding, has important implications for the interpretation of data on inbreeding effects. Some of these implications are discussed.

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SUPPRESSION OF NASAL, SKIN, AND AERIAL STAPHYLOCOCCI BY NASAL APPLICATION OF METHICILLIN *

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Untreated heavy nasal carriers of staphylococci have been reported to have staphylococci on their skin more frequently and to disseminate staphylococci in the air in larger numbers than either noncarriers or carriers of smaller numbers of these bacteria (1). If the nose is the main source of skin and aerial staphylococci, suppression of nasal staphylococci should be followed by a reduction in both aerial dissemination and skin carriage.

Of the presently available systemically administered antibiotics, only methicillin (dimethoxyphenyl penicillin) has been reported to eradicate staphylococci from nasal carriers without overt staphylococcal infections at other sites in hospitals where a large proportion of these organisms is multiple-drug-resistant (2–6). However, other investigators have reported that nasal staphylococci are eradicated less frequently and less rapidly from patients receiving methicillin for staphylococcal infections at other sites (7–9).

In the present study nasal treatment with methicillin not only suppressed nasal staphylococci but also decreased aerial dissemination of these organisms and markedly reduced skin carriage of staphylococci. Both before and after treatment, the frequency of positive skin cultures and aerial dissemination of staphylococci was proportional to the number of nasal staphylococci isolated at the time cultures were obtained.

METHODS

Quantitative nasal cultures (10) were obtained 4 or 5 times per week, January through May, 1961, from patients without staphylococcal infections on the wards of Louisville General Hospital for whom prolonged hospitalization was indicated. Patients were classified as nasal carriers if at least 2 of 3 pretreatment cultures contained coagulase-positive staphylococci. Patients who were nasal carriers were treated by random selection with one of four ointments [1] containing 0, 10, 50 or 100 mg of methicillin per g of petrolatum and lanolin ointment. A small amount of ointment was applied with the patients' fingers completely around the anterior nares 3 times per day for 1 to 2 weeks. Patients did not receive any other antimicrobial agent during this study. Skin cultures of forearm (1) and air samples using slit samplers (11) were obtained before therapy and at weekly intervals during and after therapy.

Cultures were obtained from all patients throughout their hospitalization. In patients who had not reacquired nasal staphylococci when discharged from the hospital, attempts were made to continue cultures either until staphylococci were again isolated or until at least a total of 30 days after therapy.

Cultures from patients who did not reacquire nasal staphylococci during the study were obtained in the hospital for a mean period of 23 days after treatment, with a median value of 25 days; after discharge from the hospital, additional cultures were obtained for an average period of 17 days, with a median period of 14 days.

Ten of the 14 patients who became nasal carriers after treatment did so during their hospitalization. The remaining 4 patients were followed for 10, 13, 17 and 20 days after treatment in the hospital, during which time their nasal cultures remained negative, and for an additional 33, 14, 41 and 38 days, respectively, after discharge before staphylococci were isolated.

A coagulase-positive staphylococcus from each positive culture was phage typed [2] and its susceptibility to penicillin G, tetracycline, erythromycin, chloramphenicol, kanamycin and methicillin was determined by plate-dilution methods (12). Staphylococci requiring 10 μg or more for inhibition were considered resistant. By these standards, none of the staphylococci was resistant to methicillin or kanamycin.

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[1] Ointments with and without dimethoxyphenyl penicillin were obtained from Dr. E. M. E. Morigi, Bristol Laboratories, Syracuse, N. Y.

[2] Staphylococcal bacteriophages and propagating strains were supplied by Dr. John Blair, Hospital for Joint Diseases, New York, N. Y.

TABLE I

Comparison of number of staphylococci isolated before treatment from patients who remained noncarriers as compared with patients who reacquired staphylococci

No. of staph.	Noncarriers			Reacquired		
	No. of cultures	Per cent cultures		No. of cultures	Per cent cultures	
0	3	13		3	6	
10^1–10^2	3	13	43	4	8	26
10^2–10^3	4	17		6	12	
10^3–10^4	3	13		12	25	
			35			27
10^4–10^5	5	22		1	2	
10^5–10^6	2	9		12	25	
			22			46
$>10^6$	3	13		10	21	
	23	100		48	100	

RESULTS

No differences were seen in the results obtained with the three concentrations of methicillin. All patients treated with ointments containing methicillin became noncarriers during treatment, but no change occurred in the carrier rates of patients treated with placebo ointment.

Eight of the 22 treated patients remained noncarriers for the duration of the study. Six of these 8 patients were followed for more than 30 days after therapy and the remaining 2 patients were followed for 13 and 21 days after therapy.

Staphylococci isolated from 7 of the 14 patients who became carriers after treatment were different phage types than the ones isolated from the patients before therapy, and 7 were the same phage type as that isolated before therapy. Therefore, a change in carrier status—either conversion of carriers to noncarriers or change in type of staphylococcus carried—occurred in 15 of 22 patients (68 per cent). No change in phage types occurred in patients treated with ointment without methicillin.

The time required before cultures became positive after therapy varied from 3 to 58 days, with a median value of 13 days, and no differences were seen in the time of reappearance of staphylococci in patients who acquired the same phage type as

TABLE II

Nasal carrier rates for coagulase-positive staphylococci in patients treated with topical methicillin

Treatment day		Treated patients (22 cases)		Placebo patients (7 cases)	
		No. cultured	Positive	No. cultured	Positive
			%		%
Before treatment	−4	10	90	3	100
	−3	15	100	2	100
	−2	14	86	6	100
	−1	20	95	7	100
During treatment	1	13	94	4	100
	2	6	16	4	100
	3	15	27	5	100
	4	16	6	4	100
	5	15	20	3	100
	6	17	12	7	100
	7	17	6	3	100
	8	13	8	4	100
	9	8	12	3	67
	10	12	0	4	100
After treatment	+1	12	0	3	100
	+2	9	11	4	100
	+3	14	21	5	100
	+4	13	15	4	100
	+5	8	25	1	100
	+6	10	30	2	100
	+7	10	11	3	100
	+8	8	25	2	100
	+9	8	38	1	100
	+10	12	25	3	100
	+30	13	46	2	100

TABLE III

Effect of topical methicillin on number of staphylococci in positive cultures

Treatment day		Mean of log of positive cultures	
		Treated patients (14 cases)	Placebo patients (7 cases)
Before treatment	−4	4.8	4.4
	−3	5.6	3.9
	−2	4.6	5.1
	−1	4.3	5.4
During treatment	1	3.3	5.2
	2		4.8
	3	2.2	5.3
	4	1.5	4.8
	5	2.4	5.5
	6	3.1	5.1
	7	1.3	4.2
	8	2.3	5.1
	9	2.6	5.1
	10		5.3
After treatment	+1		4.0
	+2	1.5	4.5
	+3	3.2	5.1
	+4	2.6	4.8
	+5	4.2	5.5
	+6	2.1	4.1
	+7	2.9	4.2
	+8	3.2	4.3
	+9	3.8	5.1
	+10	5.2	4.3
	+30	4.6	5.0

TABLE IV

Antimicrobial resistance of staphylococci isolated before and after treatment *

	Before treatment		After treatment	
	No. of patients	Patients	No. of patients	Patients
		%		%
Noncarriers	0	0	8	36
Sensitive	10	45	5	23
Pen. resistant	4	18	6	27
Pen., Tetra. resistant	2	9	1	5
Pen., Tetra., Eryth. resistant	3	14 (37)	1	5 (15)
Pen., Tetra., Eryth., Chloro. resistant	3	14	1	5
	22	100	22	100

* Pen. = penicillin, Tetra. = tetracycline, Eryth. = erythromycin, Chloro. = chloroamphenicol.

compared with patients from whom phage types different from those present before therapy were isolated.

Pretreatment cultures from patients who became nasal carriers after treatment contained larger numbers of staphylococci than pretreatment cultures from patients who remained noncarriers after treatment (Table I). There was no difference in the number of staphylococci isolated from pretreatment cultures of patients from whom the same phage type of staphylococci was isolated as compared with patients from whom a new phage type of staphylococci was isolated.

Topical administration of methicillin caused a rapid drop in carrier rates and in the number of staphylococci isolated from those who remained carriers (Tables II and III). After therapy, the mean carrier rate gradually increased until 46 per cent of the cultures obtained 30 days or longer after treatment contained coagulase-positive staphylococci. In patients who became carriers again, the number of staphylococci isolated from positive cultures did not equal pretreatment levels or the number isolated from patients treated with placebo ointment until 10 days after discontinuation of treatment.

Comparisons of staphylococci isolated before and after treatment showed that 37 per cent of the patients before therapy and 15 per cent of the patients after therapy were carriers of multiple-drug-resistant strains (Table IV). Forty-five per cent of the patients were carriers of a phage type 80/81 staphylococcus before treatment, but only 14 per cent were carriers of this staphylococcus after treatment (Table V).

A large proportion of air samples and skin cultures collected from treated patients before therapy contained coagulase-positive staphylococci (Table VI). During treatment the number of samples containing coagulase-positive staphylococci was very low. After therapy, staphylococci were isolated infrequently from patients who were not nasal carriers at the time skin cultures and air samples were collected, but were isolated as frequently from patients who had reacquired

TABLE V

Phage patterns of staphylococci isolated before and after treatment

	Before treatment		After treatment	
	No. of patients	Patients	No. of patients	Patients
		%		%
Noncarriers	0	0	8	36
Not typeable	5	23	7	32
Group I	1	5	0	0
Group II	4	18	2	9
Group III	2	9	2	9
Type 80/81	10	45	3	14
	22	100	22	100

TABLE VI

Frequency of positive air samples and skin cultures for staphylococci before, during, and after topical treatment with methicillin

	Air samples		Skin cultures
	Active	Inactive	
Before treatment	7/13*	4/15	5/15
During treatment	3/10	0/10	0/10
After treatment (noncarrier)	3/23	2/23	0/25
After treatment (nasal carrier)	6/10	2/10	6/13

* No. positive/no. samples.

TABLE VII

Correlation of frequency of positive skin cultures to number of nasal staphylococci

Nasal count	No. of cultures	No. positive	Positive
			%
0	40	0	0
10^1 to 10^4	12	3	25
$>10^4$	30	11	37
	82	14	17

staphylococci as from patients before treatment. No changes occurred in the frequency of positive cultures or in the number of staphylococci isolated in air samples or from skin cultures of patients treated with placebo ointment.

The frequency of positive skin cultures was greater in patients who were carriers of more than 10,000 staphylococci per nasal swab than in carriers of less than 10,000 (Table VII). In patients in whom nasal staphylococci had been eliminated by nasal ointment, no staphylococci were isolated from the skin.

No correlation was found between the frequency of positive air samples and the number of staphylococci isolated from nasal carriers when the air samples were collected while the patient rested quietly in bed (Table VIII). However, in patients in whom nasal staphylococci had been eliminated, a very low proportion of air samples contained staphylococci.

When the patients' bedding was shaken lightly for 15 seconds of the 1.5-minute collection period, positive air samples were more frequent around carriers of more than 10,000 staphylococci than around either carriers of less than 10,000 staphylococci or around noncarriers (Table IX). In addition, more than 5 staphylococcal colonies were present in 32 per cent of air samples around nasal

TABLE VIII

Correlation of frequency of positive air samples from patients at rest to number of nasal staphylococci

Nasal count	No. of samples	No. positive	Positive
			%
0	36	1	3
10^1 to 10^4	16	4	25
$>10^4$	27	6	22
	79	11	14

TABLE IX

Correlation of frequency of positive air samples from patients during activity with number of nasal staphylococci

Nasal count	Air samples	No. positive	Positive	5 Colonies/ cu ft of air	
			%	*no.*	%
0	37	5	14	1	3
10^1 to 10^4	13	5	37	2	15
$>10^4$	28	17	61	9	32

carriers of more than 10,000 colonies, in 15 per cent of air samples of carriers of less than 10,000 colonies, and in only 3 per cent of air samples around patients in whom nasal staphylococci had been completely suppressed.

Eighty-one per cent of staphylococci isolated from air samples or from the skin of nasal carriers were the same phage types and were susceptible to the same antibiotics as the staphylococci isolated from the nose at the same time. All staphylococci isolated from air samples around noncarriers were of different phage types and had different antimicrobial susceptibilities than the staphylococci isolated either from pretreatment nasal cultures or from nasal cultures obtained at a later period in those patients who reacquired nasal staphylococci. No local or generalized reactions to topical methicillin were observed during or after treatment.

DISCUSSION

In general hospitals where antibiotics are used extensively, there is a large reservoir of staphylococci resistant to penicillin G, tetracycline, erythromycin and chloramphenicol. Administration of these antibiotics to patients or personnel often eliminates sensitive staphylococci present in the nose before therapy, but treated patients acquire new strains from the hospital environment during therapy which are resistant to the administered drug and to several other antibiotics also (3, 4).

Methicillin is highly resistant to staphylococcal penicillinase and, in this hospital, no coagulase-positive staphylococci of over 3,000 strains tested were resistant to methicillin. In a previous study (2), systemic therapy with methicillin rapidly eliminated nasal staphylococci from patients without staphylococcal infections at other sites. Since there were no strains resistant to methicillin, treated patients did not acquire new strains from the hospital environment during treatment.

As shown in this study, nasal treatment with methicillin also rapidly depressed both the number of nasal carriers and the number of staphylococci present in those patients who remained carriers. Associated with the reduction in nasal staphylococci was a marked reduction in the frequency with which staphylococci could be isolated from a standard area of patients' skin and a reduction in the frequency with which staphylococci could be recovered from the air around treated patients.

In patients who became nasal carriers after treatment was discontinued, a large proportion of both skin cultures and air samples again contained coagulase-positive staphylococci. However, the strains acquired after therapy were less frequently multiple-drug-resistant or lysed by phages 80/81 than those isolated before therapy.

In this study, patients were selected who would remain in the hospital at least 3 weeks. Because of the nature of their illnesses, many had received antibiotics during previous hospitalizations and the proportion of staphylococci lysed by phages 80/81 and resistant to several antibiotics was higher than usually found in newly admitted, untreated patients (3).

If fewer strains before treatment had been drug-resistant, an increase in the number of such strains after completion of treatment might have occurred in proportion to availability of drug-resistant strains in the environment. However, selection of drug-resistant strains during treatment would require strains resistant to methicillin. Gezon, Rogers, Thompson and Hatch (13) have reported that more than one phage type of staphylococcus can be isolated from a large proportion of nasal carriers. It is possible that new phage types isolated after therapy with methicillin were present before treatment and do not represent true acquisition of new phage types. However, the close correlation between phage types of staphylococci isolated from the nose, skin, and air around individual carriers both before and after treatment suggests that changes in the predominant type of nasal staphylococci did occur.

At the present time, methicillin differs from most antimicrobial agents in suppressing nasal staphylococci, skin staphylococci, and aerial dissemination of these bacteria and in reducing the proportion of multiple-drug-resistant strains from these sources. No strains resistant to methicillin have been detected in our hospital; should resistant strains appear in large numbers, then patients treated with methicillin would be expected to acquire methicillin-resistant staphylococci from the hospital environment in a manner similar to the acquisition of strains resistant to penicillin G, tetracycline, and erythromycin by patients during treatment with these drugs. A few strains resistant to methicillin have been reported from England (14), but it is too early to predict accurately how virulent or transmissible these staphylococci will be or how frequently they may be encountered after methicillin has been used for a longer period of time.

The relative role of nasal carriers of staphylococci in initiating staphylococcal infections has not been completely established. However, a number of epidemics of staphylococcal diseases has been ascribed to a simple nasal carrier (15–17). Also, in two studies (18, 19) patients who were nasal carriers had a higher incidence of postoperative staphylococcal infections than patients who were noncarriers, and the increased incidence of infection was due to staphylococci of the same phage types as those present in the nose before the infection occurred.

It has rarely been possible to prove that infections were caused by aerial dissemination or by direct contact with skin staphylococci. However, studies by Wolinsky, Lipsitz, Mortimer and Rammelkamp (20) have shown that, at least in one nursery, direct contact by hands was very important in dissemination of staphylococci. Regardless of the method of transfer, the present studies suggest that a decrease in the number of nasal staphylococci or elimination of the nasal carrier status should reduce both the risk of aerial dissemination and of infections from skin staphylococci.

This study was designed to test the correlation between nasal staphylococci and dissemination of these organisms onto the skin and into the air. Until the role of nasal carriers in initiating staphylococcal infections can be established and until the risks of selecting methicillin-resistant staphylococci with either topical or systemic therapy are known, topical treatment of nasal carriers with methicillin as a means of reducing staphylococcal infections must remain an experimental approach.

SUMMARY

Nasal administration of methicillin rapidly decreased both the number of nasal carriers of staphylococci and the number of staphylococci isolated from patients who remained carriers.

Approximately one-third of treated patients reacquired staphylococci of the same phage types as those present before therapy; in one-third of the patients different phage types of staphylococci were isolated after treatment; and one-third remained noncarriers.

Staphylococci isolated after treatment were less frequently multiple-drug-resistant than those isolated before treatment.

Depression of nasal staphylococci was followed by a rapid reduction of skin staphylococci and of aerial dissemination of these organisms.

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WATER EXCRETION IN POTASSIUM-DEFICIENT MAN *

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Kaliopenic nephropathy is now a well recognized entity in which diminished ability to concentrate urine is a prominent clinical feature (1–4). Most instances are reported in patients with an aldosterone-producing tumor or chronic diarrhea (5), and the defect in urine concentrating ability was well established when potassium deficiency was recognized. Usually the magnitude and duration of the potassium deficit could be only indirectly inferred.

Most studies of renal anatomy and function during potassium deficiency have been performed in animals, but it is generally assumed that man behaves similarly, and *a*) anatomic lesions in the collecting ducts and inner medulla cause the functional incapacity (3), *b*) the severity of these lesions reflects the magnitude of potassium depletion (2, 3, 6), and *c*) the functional and possibly the anatomic aberration is reversible if potassium is repleted (1, 3, 5).

Extrapolation of data obtained in the rat to limited experience in man is uncertain. The anatomic lesions of the kidney seem quite different in rats (7–11) and men (2, 12–14). Human lesions are often maximal in the proximal convolution rather than in the collecting system, and the vacuolated renal tubular epithelium usually seen in man (and dog) may be absent or minimal in the rat. Secondly, because potassium deficiency and the circumstances which cause it threaten health, serial study of the same untreated patient is usually precluded, and correlation of renal functional studies with the potassium deficit is of limited utility. Finally, focal scarring occurs in kidneys of animals maintained for even brief periods in the potassium-depleted state (15–17), and the reversibility of function with potassium repletion has been questioned (3, 13, 18).

The studies reported herein describe certain experiences with experimental potassium depletion in man; they attempt to quantify in time the extent of the alteration in renal concentrating ability during progressive potassium depletion and its resolution during potassium replenishment. A temporal dissociation between the ability to concentrate the urine maximally during hydropenia, and the ability to conserve water during acute solute loading was found. Although permanent impairment of renal function was not observed in these studies, it is felt that this was fortuitously related to the relatively limited magnitude or duration of potassium depletion, and in view of the rat experience reported after these studies were instituted (17), which indicates that permanent renal scarring may result from even brief potassium depletion, we would hesitate to induce again severe potassium depletion in man.

METHODS

Nine male patients participating in these studies were convalescent from orthopedic procedures, and free of cardiovascular, renal and endocrine disease by history, physical examination and the usual laboratory screening procedures. Experimental potassium depletion was produced by feeding a constant liquid diet, adequate in calories, protein and vitamins, but containing less than 0.1 mEq of potassium per kg body weight. Since the diet was made up of natural foods, it is likely that other trace elements were not seriously deficient. The diet could maintain nitrogen balance in adult man, and with added KCl, could promote normal growth in young rats. Sodium intake was kept constant, generally at 1 mEq per kg of body weight, but in certain studies was modified by protocol design. In some studies protein intake was kept low during the initial period of potassium depletion, then quadrupled without increasing potassium intake. Cumulative losses of potassium were monitored from analyses of urine and feces only, and are minimal rather than precise estimates of degree of potassium depletion. Their magnitude was again estimated during repletion, and in each instance was confirmatory within 15 per cent. Measured amounts of distilled water were given *ad libitum*.

Each subject was first observed for 1 week on the metabolic ward. KCl, 1.0 mEq per kg body weight, was given

* Material presented in abstract form to the Southern Section, American Federation for Clinical Research, January, 1960 (Clin. Res. 1960, **8**, 64).

with the potassium-depletion diet during this period, and control studies of concentrating ability during dehydration, water reabsorption during mannitol diuresis, the response to a water load, and vasopressin sensitivity were performed. The test diet was then continued without potassium supplements for 20 to 35 days, and these studies were repeated at intervals. Serial concentration tests twice weekly were feasible in six subjects throughout the depletion and repletion periods; observation was less complete in the others. Three or more mannitol infusions were performed in five potassium-depleted subjects; in three others only a control study and a single study during potassium depletion were performed. Similarly, serial weekly studies of water diuresis were feasible in only four of the subjects, although several studies during the potassium-depletion period were performed in the others.

Urinary concentrating ability was assessed after overnight dehydration both with and without Pitressin (vasopressin). Although a more concentrated urine can be achieved by normal men by prolonging or intensifying the period of dehydration, or by increasing the protein content of the diet (19, 20), this simple technique gives closely reproducible results in any single individual (average variation from the mean of two or more tests: normals, 4.2 per cent; renal disease with hyposthenuria, 6.9 per cent). Water reabsorption was examined during osmotic diuresis produced by infusing 10 per cent mannitol during dehydration. Dehydration tests and osmotic diuresis were usually performed in tandem; i.e., after two or three hourly urines were collected, the mannitol infusion was started and infusion rate slowly raised until maximal urine flow was achieved. Sufficient vasopressin was added to the infusion (in excess of 200 μU per hour) to insure maximal antidiuretic hormone (ADH) activity. Maximal water reabsorption (T^cH_2O, the difference between osmolar clearance and urine flow) was arbitrarily selected as the average value obtained in three to five collection periods agreeing within $\pm$ 10 per cent. These generally were obtained at intermediate rates of solute excretion (C_{osm}, 15 ml per minute), for with more rapid rates of mannitol infusion T^cH_2O appeared to decrease irregularly.

Water diuresis was produced by giving 20 ml per kg of tap water, or by giving a sustained load of 1.0 L. The adequacy of diuresis after a water load was evaluated from time course and magnitude of peak urine flow, minimal urine osmolality, and gross water balance after 2 and 3 hours. Often minimal urine osmolality was also observed during sustained water loads. The reproducibility of such data even in the same subject on constant diet was less than ideal, reflecting undoubtedly the uncontrolled differences in antecedent bodily hydration and fluctuating renal hemodynamics. At least 2 days separated tests of dilution and concentration.

Sensitivity to exogenous vasopressin was examined at intervals during progressive potassium depletion. Each subject was titrated by determining the minimal amount of vasopressin given intravenously during water diuresis which could induce urine to become more concentrated than plasma. Then twice this amount of vasopressin was administered in successive studies using a fresh ampule of the same lot for each study to obviate the problem of deterioration after dilution. Two different lots were used which differed by cross assay in normal man by 50 per cent, so that cited dosage administered is only a rough approximation.

All urines were collected by spontaneous voiding. Urinary sediment was examined at frequent intervals. In five subjects, quantitative sediment counts were performed daily on overnight specimens. Fleeting mild albuminuria or increased cellular elements were seen in most cases, but rarely in consecutive specimens. Three subjects who had increased numbers of white cells in their urinary sediment on several occasions were studied more intensively, but repeated urine cultures revealed no significant bacilluria.

Chemical methods employed have been previously described (21). Osmolality of plasma and urine was estimated with a Fiske osmometer by comparing the freezing point depression to that of standard solutions of sodium chloride.

RESULTS

Potassium-restricted subjects lost their ability to concentrate urine normally (Figure 1). Significant hyposthenuria was apparent as early as the fourth day in one subject and common to all the subjects by the second week. Generally the impairment of concentrating ability was slight until approximately 200 mEq of potassium had been lost. After approximately 3 weeks of potassium restriction (deficit, 350 mEq), the degree of hyposthenuria became relatively constant although potassium restriction continued, and even in the most severe potassium depletion, 24-hour urine

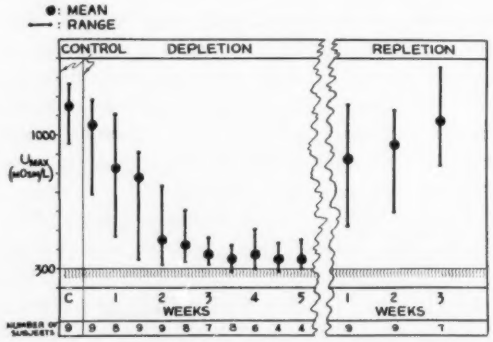

FIG. 1. URINE CONCENTRATION DURING HYDROPENIA IN POTASSIUM DEPLETION. The mean and range of maximal urine concentration examined in 9 subjects is depicted. The mean U_{max} is significantly ($p < 0.05$) different from control by the tenth day.

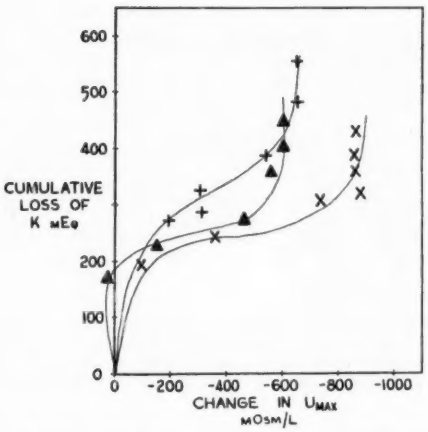

FIG. 2. RELATIONSHIP BETWEEN URINE CONCENTRATING ABILITY AND THE MAGNITUDE OF THE POTASSIUM DEFICIT.

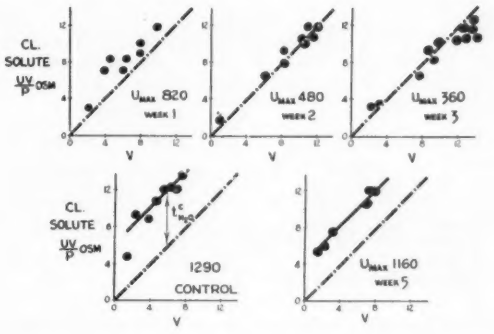

FIG. 3. WATER REABSORPTION DURING OSMOTIC DIURESIS IN PROGRESSIVE POTASSIUM DEFICIENCY. Serial studies in a single individual are depicted. The U_{max} refers to concentration during hydropenia obtained before starting the mannitol infusion. The study during Week 5 was performed after repletion; external balance of potassium was + 80 mEq.

was generally more concentrated than plasma. *Linear* correlation of the loss of urine concentrating ability with the cumulative loss of potassium was low ($R = 0.55$, $p > 0.1$), and such correlation seemed appropriate only in the range of potassium deficit of 200 to 400 mEq. At values above and below these limits, changes in potassium balance were little reflected in changes in maximal urinary concentration (U_{max}). The diphasic curves shown in three subjects (Figure 2) were typical.

Maximal water reabsorption during osmotic diuresis fell abruptly as potassium was withdrawn from the diet (Figure 3, Table I). The concentrating defect was prominent at high rates of solute excretion in subjects who retained considerable concentrating ability during simple dehydration when the rate of solute excretion was low. Thus two subjects could concentrate urine to 800 mOsm per L during dehydration, but maximal T^cH_2O was < 3.0 ml per minute; four subjects who could concentrate urine to twice plasma osmolality could not reabsorb 1 ml per minute T^cH_2O.

As the potassium deficit was increased further, the U/P osmotic gradient during hydropenia could be maintained only when the rate of solute excretion was low. After more than 300 mEq of

TABLE I

Water reabsorption in potassium-depleted subjects *

Control	Week	Potassium depletion 1	2	3	4	Recovery 2	4+
U_{max} 1,140 ± 80 (*mOsm/L*) [9]		801 ± 110 [9]	410 ± 40 [8]	340 ± 20 [6]	328 ± 18 [5]	960 ± 42 [9]	
Max. T^cH_2O 5.6 ± 0.9 (*ml/min*) [9]		1.2 ± 0.8 [8]	0.2 ± 0.6 [5]	−0.8 ± 0.9 [6]	−0.1 ± 0.4 [3]	0.9 ± 1.2 [4]	5.3 ± 1 [5]
K balance (*mEq*)		−210 ± 62 [9]	−295 ± 32 [9]	−354 ± 60 [8]	−428 ± 26 [3]	60 ± 15 [9]	

* The values reported (mean ± standard deviation) are arbitrarily grouped by weeks of depletion. When more than one study was performed during a single week the values obtained were averaged. The number of patients studied in each period is shown in brackets. The numerical values assigned to T^cH_2O during potassium restriction are arbitrary and conceptually artifactual, since in most studies no apparent plateau was achieved and urine often became progressively less concentrated as solute load was raised. The calculated T^cH_2O values that were observed at the same range of solute excretion rates obtained in the control study were averaged. This is better depicted in Figure 3 and discussed further in the text. The repletion studies are timed from the onset of repletion. Although potassium was given at different rates and retention of potassium varied, all subjects had a calculated net positive balance of potassium by 2 weeks.

TABLE II

Parameters of water diuresis compared in seven subjects at approximately equivalent deficits of potassium (250 to 300 mEq) *

	Control		Deficit 250–300 mEq						
	Mean	Range	Subject no.: I	II	III	IV	V	VI	VII
			Day of study: 16	18	20	21	22	26	28
Maximal flow (*ml/min*)	14.2	10.6–22.3	13.8	8.3†	14.7	16.2	21.9	9.4†	11.1
Minimal osmolality (*mOsm/L*)	69	60–89	72	120†	65	60	64	71†	88
"Lag time" (*min*)	60	35–110	48	80†	90	75	93	65†	
% Excreted at 2 hrs	81†	62–106		82				60†	

* The day of study is shown to give some estimate of the rate of development of potassium deficiency. "Lag time" refers to the time elapsed between water load and peak urine flow. Excretion at 2 hours was not corrected for insensible losses of about 50 ml/hour. All data were obtained during sustained water loading except those designated by † to denote a single water load. The data demonstrate normal water diuresis at a time when urine concentrating ability was severely reduced.

potassium had been lost, maximal water reabsorption in three of six subjects in whom an osmotic diuresis was induced was actually "negative." Although urine during hydropenia was in each instance at least 20 per cent more concentrated than coincident plasma, the osmotic gradient was rapidly lost with increasing solute excretion, and urine became more dilute than plasma. Potassium replenishment caused a prompt return in the ability to concentrate urine during hydropenia. This was not true of water reabsorption during mannitol loading and maximal T^cH_2O was severely reduced when U_{max} had already returned to normal (Table I). Two of the five subjects studied 1 month after repletion still had a low T^cH_2O (3.2 and 3.6 ml per minute). Three months later, the opportunity to re-examine one of these subjects and two of the others revealed no abnormalities of renal function.

Water diuresis was little affected by moderate potassium deficiency and a seemingly normal response to ingested water was obtained in subjects whose capacity for urine concentration had already deteriorated (Table II). When potassium deficiency progressed beyond 350 mEq, abnormal water excretion became evident (Table III). The response to intravenous loads of a dilute fructose solution was also abnormal at this time, indicating that impaired intestinal absorption was not the decisive factor. The abnormal water diuresis was not improved by salt loading except in a subject whose previous sodium intake had been restricted parallel to potassium restriction, and who never achieved a normal response to water loading even in the first week. Although consistent changes in creatinine clearance were not found during potassium deficiency, significant changes in glomerular filtration were not ruled out.

Increasing resistance to vasopressin became evident as potassium deficiency progressed. Yet this did not always closely parallel loss of concentrating ability, and two of the subjects who could still concentrate their urine to 400 to 500 mOsm per L during dehydration continued to excrete very dilute urine during water diuresis despite vasopressin administration (Figures 4 and 5). While it is attractive to consider that these data would substantiate a dual process of water reabsorption (25, 26), one partly independent of ADH action, creatinine clearance and

TABLE III

Abnormal water diuresis in three of five subjects studied when the potassium deficit exceeded 350 mEq

Deficit (*mEq*):	351	382	396	422	461	524
Subject:	III	I	VII		VI	V
Maximal flow (*ml/min*)	6.2	11.8	2.4	1.4	19.2	1.6
Minimal osmolality (*mOsm/L*)	185	82	232	201	76	206
"Lag time" (*min*)	80	75			45	
% Excreted at 2 hrs	22	92	8	10	102	11

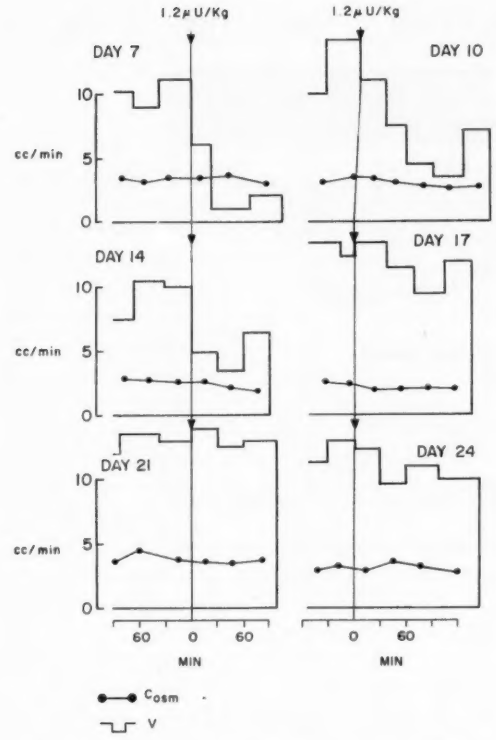

FIG. 4. VASOPRESSIN RESPONSIVENESS IN POTASSIUM DEFICIENCY. The measured potassium deficit was approximately 200 mEq by Day 10 and 320 mEq by Day 21. The study on Day 24 was performed after 200 mEq had been given, replacing more than half the cumulative deficit. This lot of vasopressin had probably lost considerable activity. See text.

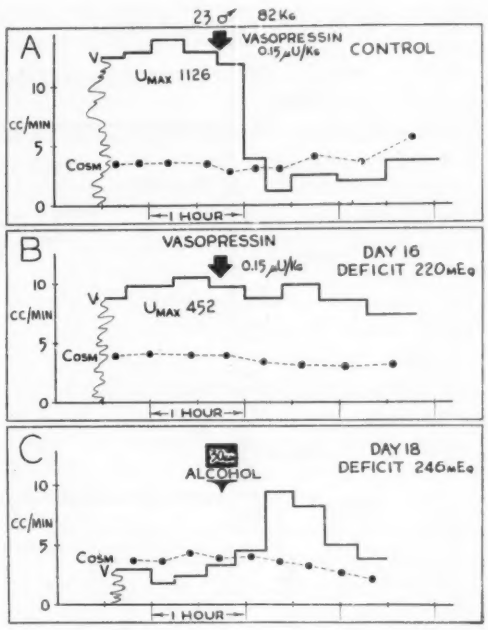

FIG. 5. "WATER TYPE" DIURESIS AFTER ETHYL ALCOHOL INGESTION IN A POTASSIUM-DEPLETED SUBJECT RESISTANT TO VASOPRESSIN. Free water clearance is depicted as the difference between C_{osm} and V.

hence probably glomerular filtration rate invariably increased during mannitol infusion, and it is difficult to explain the effect of ethyl alcohol in the moderately potassium-depleted subject (Figure 5). The "water-type" diuresis evident after

TABLE IV

Effect of urea on T^cH_2O in potassium-depleted dogs *

	Rate of solute excretion		
	1,000–2,000	2,000–3,000	3,000+
	μOsm/min	*μOsm/min*	*μOsm/min*
Normal	3.6 to 4.2	3.9 to 4.4	3.4 to 5.2
Normal + urea	2.9 to 4.6	3.8 to 5.2	4.7 to 6.5
K-depleted	−0.1† to 0.3	−1.2† to 0.5	−1.1† to 0.4
K-depleted + urea	−0.4† to 0.5	−1.0† to 0.1	−1.2† to 0.1

* Range of T^cH_2O during osmotic diuresis in the same dogs studied before and after potassium depletion. The potassium-deficient diet was fed for 5 weeks prior to study. Cumulative negative balance was in excess of 90 mEq (approximately ¼ normal body content) and moderate hypokalemic alkalosis was present. The infusion rate of 10% mannitol was increased until urine flow exceeded 10 ml/min for three successive periods. Then 50 g of urea was instilled into the stomach and rate of mannitol infusion slowly reduced to observed T^cH_2O over a wide range of solute loads. The post-urea periods are limited to those in which urea excretion had increased more than twice. The data from 3 dogs are amalgamated.

† A negative T^cH_2O indicates urine was more dilute than plasma.

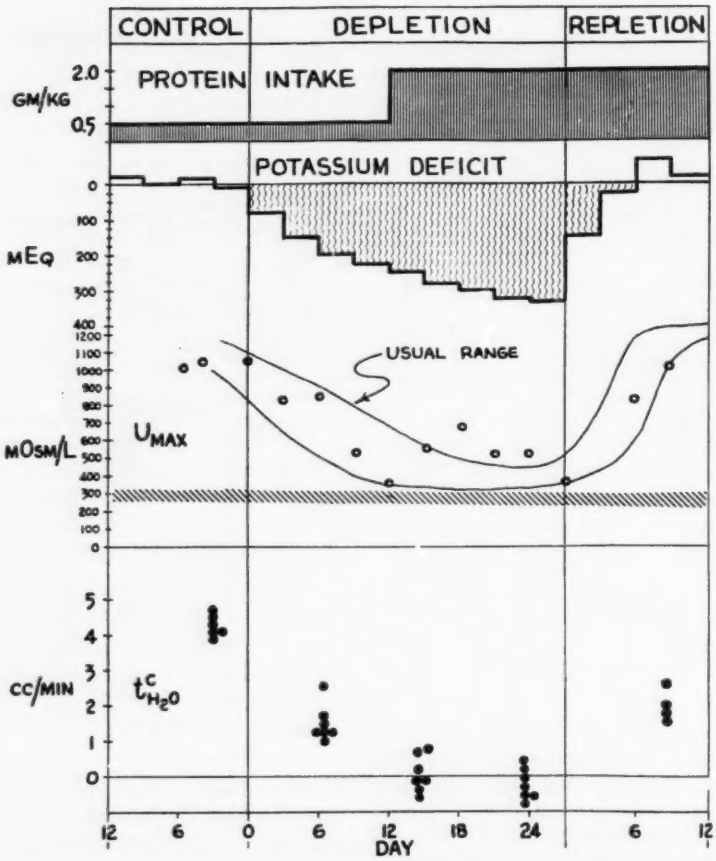

FIG. 6. EFFECT OF PROTEIN FEEDING ON WATER CONSERVATION DURING POTASSIUM DEFICIENCY. Not shown is the balance of nitrogen and phosphorus which became positive. The relative potassium deficit was probably aggravated (see text). Serum potassium fell from 3.8 to 2.6 mEq per L when protein intake was quadrupled in this subject, but remained unchanged in a similar study on another subject.

alcohol suggests that some ADH was previously present and acting on the renal tubule, since the main action of alcohol on water balance seems to be inhibition of ADH release (22). This is further implied by the absence of hypotonic polyuria which might be expected if total insensitivity to ADH was present (23, 24).

Protein feeding or urea administration may increase apparent urine concentrating ability both in normal subjects and in some patients with hyposthenuria due to renal disease (25). The effect of increased loads of urea on the hyposthenuria of potassium deficiency was examined in three subjects. In a subject whose dietary protein was increased while potassium restriction continued, maximal urinary concentration was more than 100 mOsm per L higher on the high protein intake, although the degree of potassium depletion had increased 100 mEq (Figure 6). A second subject, more severely depleted of potassium, similarly increased U_{max}, but in an another subject with hyposthenuria and potassium depletion of over 300 mEq, 3 days of urea feeding did not appreciably alter concentrating ability. Feeding urea during mannitol infusion, which increases apparent maximal water reabsorption in normal dogs, and, under special circumstances, in man (25, 26), did not increase water reabsorption in

potassium-depleted dogs (Table IV), or in a potassium-depleted subject.

DISCUSSION

The inability to concentrate urine normally that attends potassium depletion had two main characteristics: *1*) U_{max} fell slowly and reached a minimum only when considerable potassium had been lost from the body; yet at the extreme deficits of this study, urine could be concentrated to levels higher than plasma osmolality. *2*) T^cH_2O deteriorated abruptly when potassium was restricted and, even with mild potassium deficits, increasing solute load abolished the U/P osmotic gradient despite maximal ADH activity. While massive solute loading may induce a progressive fall in negative free water clearance and even hypotonic urine in dehydrated dogs, such occurrence in man is unusual, and was unexpected with this moderate degree of solute loading. Similar mannitol infusions in isosthenuric patients with advanced renal disease have not, in our experience, induced urine more dilute than plasma.

A readily demonstrable defect in renal tubular function occurred with degrees of potassium depletion tolerated by other body cells with apparent impunity (27–29). This may be ascribed to an apparent critical vulnerability of the renal tubular epithelium to potassium deficiency; however, comparable techniques to examine other body cells are lacking. Urine concentrating ability was invariably compromised while serum potassium concentration remained within the normal range. The failure of serum concentration of potassium consistently to reflect body stores has been frequently pointed out (2, 30–32) and is probably related at least in part to the level of sodium intake (33, 34).

The intensity and significance of a given potassium deficit must undoubtedly include a temporal factor so that the length of time a given potassium deficit is maintained and the rate at which it developed must probably qualify the pathophysiologic response (22–24, 30). We have tried to induce a mild but acute potassium depletion by reducing potassium content of the dialysis bath of a patient being hemodialyzed for barbiturate poisoning. Water reabsorption was measured during continuous mannitol diuresis. A measured depletion of over 250 mEq of potassium failed to alter water reabsorption acutely.

The mechanism by which the defect is produced is not entirely clear but certain possibilities can be considered. *1*) Osmotic diuresis. The inability to concentrate adequately is common to many types of anatomic injury which reduce effective renal mass. Fewer nephrons processing a given solute load produce urine similar to that obtained during osmotic diuresis. The hyposthenuria of potassium depletion cannot be readily ascribed to increased osmotic load per nephron unit, as filtration rate was relatively unimpaired, and the concentrating defect was evident even when the solute load was reduced by feeding a diet restricted in sodium or protein as well as in potassium. *2*) Animal studies indicate that there is a lessened hypertonicity of tissue samples taken from the region of the papilla in potassium-depleted animals (35). This may indicate either that less solute has been reabsorbed from the tubular urine or that less solute has been retained *in situ* by the countercurrent system of the medullary vascular bundles. These data indicate that kaliopenic isosthenuria can not be attributed *solely* to ADH refractoriness, unless it is assumed that the permissive action of ADH on water diffusion indirectly regulates medullary blood flow and hence solute retention. A reduced solute concentration in the medulla may prevent efficient operation of the countercurrent multiplier system of the loops of Henle and the collecting ducts by reducing the rate of transfer of water across the tubular cell (36–39).

Even if one assumes that solute reabsorption is altered in potassium deficiency, the source of medullary solute remains unclear. In potassium-deficient rats unable to concentrate urine normally, micropuncture samplings from the distal convolution failed to show altered concentration (38, 40). While this may localize the final concentrating defect in potassium-deficient rats to a more terminal segment of the nephron, possibly the collecting ducts, the possibility that reduced reabsorption of sodium occurred in the loop of Henle or even in the proximal convolution is not entirely negated. Perhaps flow rate rather than concentration of tubular urine was altered. Restrained diffusion of water could have maintained normal plasma/tubular fluid osmotic gradients even while

flow rate was increased so that the volume of water reaching the collecting ducts may have been in excess of concentrating capacity.

The subjects of this report could normally dilute their urine at a time when their concentrating ability had already deteriorated, implying that solute reabsorption during water diureses (presumably the ascending limb of Henle's loop) was adequate for the normal genesis of free water. The redistribution of body fluids with sodium retention, especially the increased extracellular volume that attends potassium deficiency (*27–29*) if sodium is not restricted, could be expected to alter the excretion of a water load. Extrarenal factors, such as production and renal responsiveness to aldosterone and changes in body fluid volumes, may be produced by potassium depletion and may exert unknown effects on these experiments. Furthermore, extrapolation from water diuresis to hydropenic states may not be valid.

It is attractive to consider the proximal reabsorption of sodium as critical to the diminished concentrating ability in potassium deficiency, for such a concept would not relegate to insignificance the distinct anatomic alterations in the proximal convolution; but, although phenol red and para-aminohippurate are poorly excreted in potassium deficiency, evidence for other "proximal" dysfunction is meager. Salt wastage during rigid sodium restriction might be expected if proximal solute reabsorption were seriously impaired in potassium deficiency, but this is not usual in potassium-depleted states. We would suspect that the defect is a consequence of diminished reabsorption of sodium rather than of impaired equilibration of water. That the hydropenic potassium-deficient subject could still concentrate urine when solute load was kept low implies that the countercurrent system was operating effectively (albeit at limited capacity) and that the countercurrent function must be to some degree independent of the tonicity within the medulla. Reduction in the osmotic gradient between urine and interstitial fluid of the medulla may be adequate to establish a relatively high urine concentration during dehydration when flow rate is very low, but might be inadequate to maintain an effective gradient during osmotic diuresis when flow rate is greatly increased.

Both reduction in volume of filtrate and establishment of a high osmotic ratio define the conservation of urinary water. The retention of the latter capability at low solute loads may reflect the methodologic inadequacy wherein high solute loads are needed to "unmask" the altered equilibration of water. It was not possible within such protocol to compare widely different rates of solute excretion at identical filtration rates, and the temporal "dissociation" may be an unavoidable artifact reflecting renal hemodynamic changes.

Increased retention of nitrogen and phosphorus that occurred with protein feeding implies storage of protoplasmic nitrogen without potassium, so that the potassium deficiency may have been aggravated at the cellular level, and the somewhat greater concentrating ability that obtains with protein feeding becomes even more significant. It appears that the increased contribution of urea to the peritubular osmolality may overcome in part the deficit in medullary tonicity and hence increases the concentration attained by the final urine.

The effect of urea on the concentrating process may be qualified by the coincident osmotic load, since we were unable to increase water reabsorption by giving urea to potassium-depleted dogs at the height of mannitol diuresis. The failure of urea feeding, which should have increased urea excretion without altering relative potassium deficiency, is unexplained.

SUMMARY

Serial studies of urine concentrating ability were performed in man during progressive potassium depletion. A temporal dissociation between the ability to concentrate urine during hydropenia and the capacity to reabsorb water during osmotic diuresis was found. U_{max} was resistant to potassium depletion until about 200 mEq of potassium had been lost, progressively fell as the potassium deficit was doubled, and then reached a threshold hypertonic to plasma despite continuing potassium depletion. Free water reabsorption during mannitol diuresis fell abruptly with potassium restriction, and approached zero at a time when appreciable concentrating ability was retained. With more severe potassium deficiency, urine became hypotonic to plasma with solute loading. The capacity to dilute urine after water loading was retained. Some considerations of the

mechanism whereby potassium deficiency may alter water conservation are discussed.

ACKNOWLEDGMENTS

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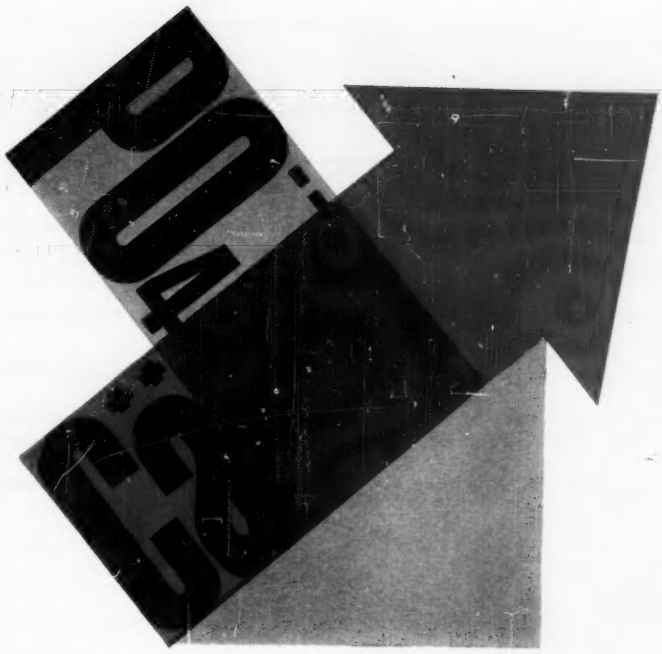

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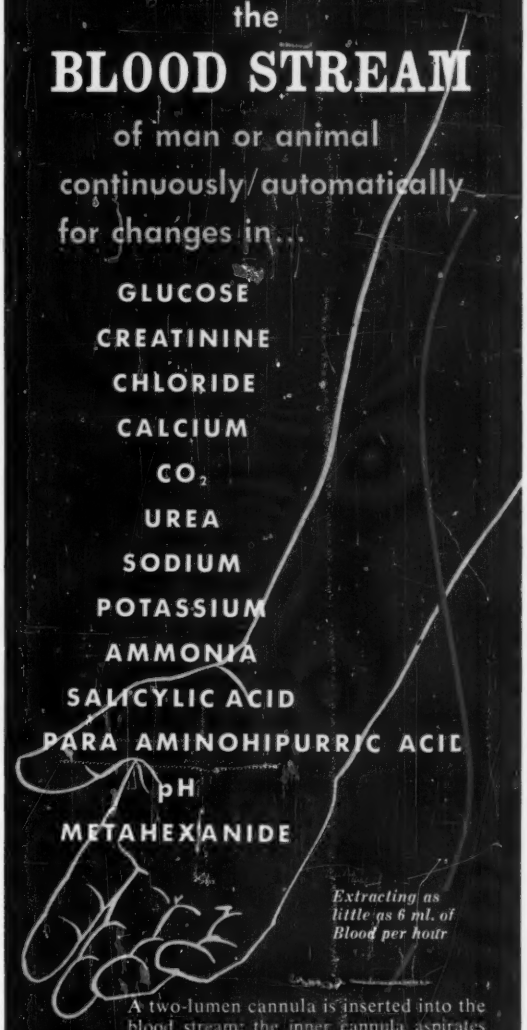

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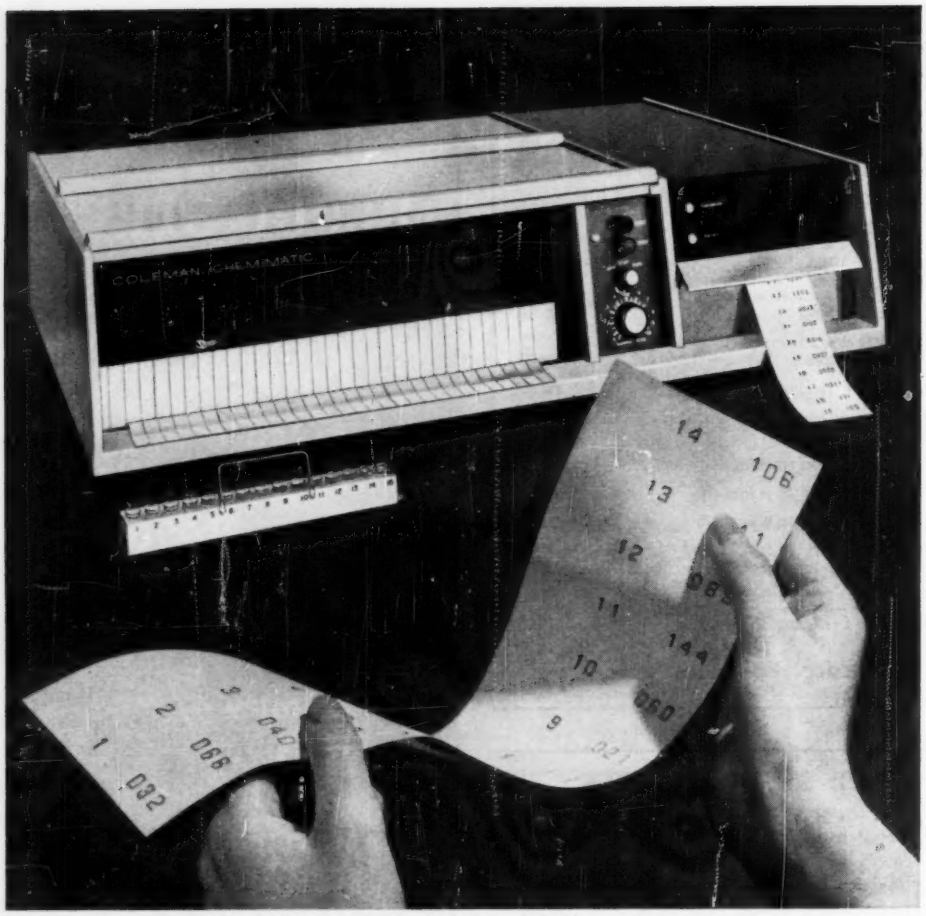

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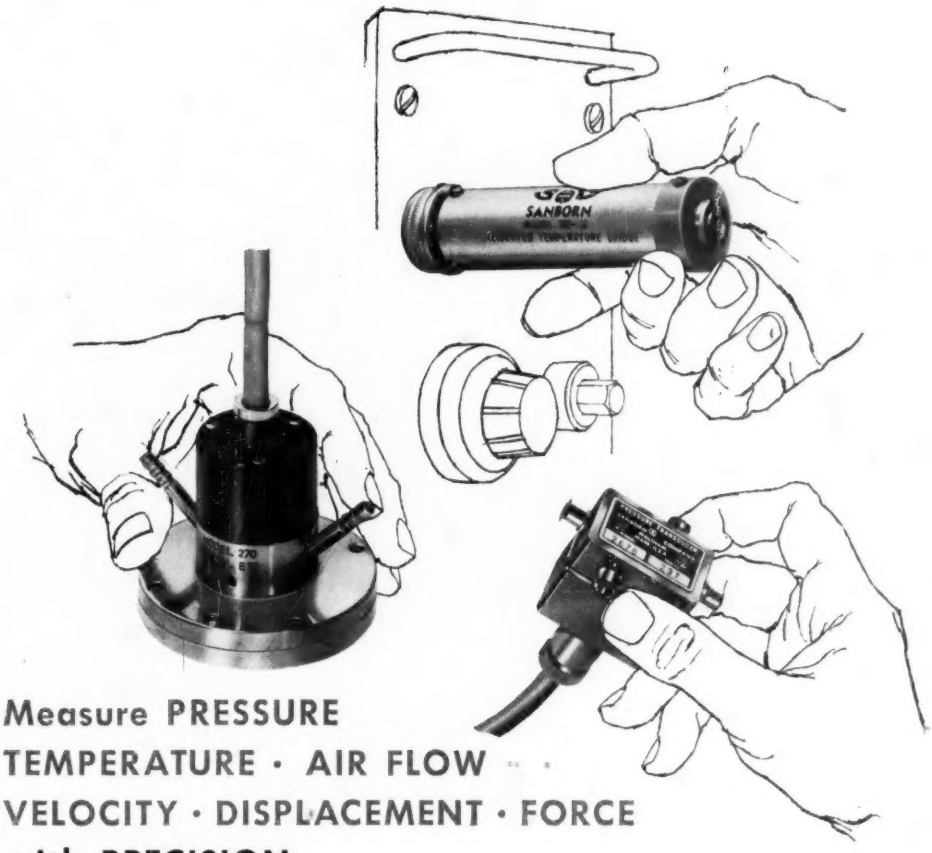

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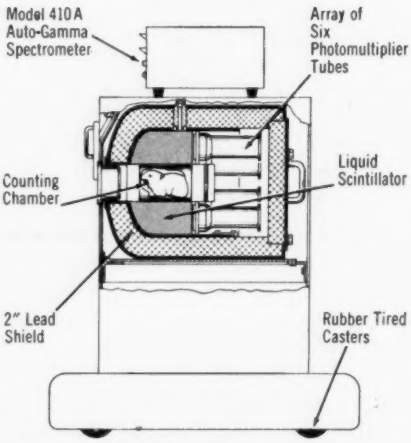

DIAGRAM—**ARMAC** SCINTILLATION DETECTOR WITH SPECTROMETER

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